Trastuzumab and Chemotherapy:

In Vitro Cytotoxicity Against HER2-Positive Breast Cancer Cell Lines*

Synergistic (CI<1)		Additive (CI~1)		Subadditive (CI>1)	
Vinorelbine	0.34	Doxorubicin	0.82-1.16	Fluorouracil	2.87
Docetaxel/carboplatin	0.34	Paclitaxel	0.91		
Docetaxel	0.41	Epirubicin	0.99		
Etoposide	0.54	Vinblastine	1.09		
Cyclophosphamide	0.57	Methotrexate	1.36		
Paclitaxel/carboplatin	0.64				
Thiotepa	0.67				
Cisplatin	0.67				
Liposomal doxorubicin	0.7				
Gemcitabine	<0.5->5 (variable, dose-dependent)				

d on a Combination Index (CI) score from multiple drug-effect analysis at fixed molar ratios.

m et al. Oncogene. 1999;18:2241; Pegram et al. Semin Oncol. 2000;27(suppl 11):21; Slamon and Pegram. Semin 2001;28(suppl 3):13; Hirsch et al. Clin Breast Cancer. 2002;3(suppl 1):S12.

Inhibitory effects of combinations of HER-2/*neu* antibody and chemotherapeutic agents used for treatment of human breast cancers

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Previous studies have demonstrated a synergistic interaction between rhuMAb HER2 and the cytotoxic drug cisplatin in human breast and ovarian cancer cells. To define the nature of the interaction between rhuMAb HER2 and other classes of cytotoxic drugs, we applied multiple drug effect/combination index (CI) isobologram analysis to a variety of chemotherapeutic drug/rhuMAb HER2 combinations *in vitro*. Synergistic interactions at clinically relevant drug concentrations were observed for rhuMAb HER2 in combination with cisplatin (CI = 0.48, $P = 0.003$), thiotepa (CI = 0.67, $P = 0.0008$), and etoposide (CI = 0.54, $P = 0.0003$). Additive cytotoxic effects were observed with rhuMAb HER2 plus doxorubicin (CI = 1.16, $P = 0.13$), paclitaxel (CI = 0.91, $P = 0.21$), methotrexate (CI = 1.15, $P = 0.28$), and vinblastine (CI = 1.09, $P = 0.26$). One drug, 5-fluorouracil, was found to be antagonistic with rhuMAb HER2 *in vitro* (CI = 2.87, $P = 0.0001$). *In vivo* drug/rhuMAb HER2 studies were conducted with HER-2/*neu*-transfected, MCF7 human breast cancer xenografts in athymic mice. Combinations of rhuMAb HER2 plus cyclophosphamide, doxorubicin, paclitaxel, methotrexate, etoposide, and vinblastine *in vivo* resulted in a significant reduction in xenograft volume compared to chemotherapy alone ($P < 0.05$). Xenografts treated with rhuMAb HER2 plus 5-fluorouracil were not significantly different from 5-fluorouracil alone controls consistent with the subadditive effects observed with this combination *in vitro*. The synergistic interaction of rhuMAb HER2 with alkylating agents, platinum analogs and topoisomerase II inhibitors, as well as the additive interaction with taxanes, anthracyclines and some antimetabolites in HER-2/*neu*-overexpressing breast cancer cells demonstrates that these are rational combinations to test in human clinical trials.

Keywords: HER-2/*neu* (c-*erb*B-2); chemotherapy; breast cancer; multiple drug effects analysis; synergy

Introduction

Overexpression of p185[HER-2/*neu*], resulting from amplification of the HER-2/*neu* gene, is associated with poor clinical outcome in 25–30% of carcinomas of the breast (Slamon *et al.*, 1987), as well as in other human malignancies (Semba *et al.*, 1985; Slamon *et al.*, 1989; Berchuck *et al.*, 1991; Yonemura *et al.*, 1991; Hetzel *et al.*, 1992; Lukes *et al.*, 1994; Press *et al.*, 1994; Saffari *et al.*, 1995). The murine monoclonal antibody 4D5 has specificity for a juxtamembrane epitope in the extracellular domain (ECD) of the p185[HER-2/*neu*] protein (Fendly *et al.*, 1990) and is capable of eliciting an antiproliferative effect against murine cells transformed by HER-2/*neu* as well as human malignant cell lines and xenografts overexpressing this oncogene (Chazin *et al.*, 1992). Importantly, this growth inhibitory effect is specific for cells with HER-2/*neu* overexpression and does not occur with cells expressing normal amounts of the protein (Hudziak *et al.*, 1989; Chazin *et al.*, 1992). A recombinant, humanized form of 4D5 (rhuMAb HER2) has been generated by inserting the complementary-determining regions (CDRs) of 4D5 into the framework of a consensus human IgG₁ (Carter *et al.*, 1992). When compared to murine 4D5, rhuMAb HER2 exhibits a stronger binding affinity for p185[HER-2/*neu*] but has similar specific antiproliferative activity against HER-2/*neu*-overexpressing cell lines and xenografts.

To determine how best to use this antibody both as a single agent and in combination with established cancer therapeutics, we undertook a series of studies to evaluate its inhibitory effects in preclinical models *in vitro* and *in vivo*. These studies were based on a previous report of enhanced activity of cisplatin (CDDP) when used in combination with antibodies directed against the epidermal growth factor receptor (EGFR) (Aboud-Pirak *et al.*, 1988). Initial studies showed that when used in combination with the drug CDDP, 4D5, rhuMAb HER2, as well as other anti-HER-2/*neu* antibodies, potentiate cytotoxicity of the chemotherapeutic by decreasing DNA repair activity following CDDP-induced DNA damage (Hancock *et al.*, 1991; Pietras *et al.*, 1994). This effect, termed receptor enhanced chemosensitivity (REC), specifically targets HER-2/*neu*-overexpressing cells and has no effect on cells or tissues expressing physiologic levels of the gene. The interaction between 4D5 and CDDP in inhibiting HER-2/*neu*-overexpressing cell lines has been shown to be synergistic resulting in a two-log increase in CDDP-induced cytotoxicity as well as pathologic complete remissions in experimental animals bearing HER-2/*neu*-overexpressing human breast cancer xenografts (Pietras *et al.*, 1994).

Synergy, as it applies to drug-drug interactions, is defined as a combination of two or more drugs which achieves a therapeutic effect greater than that expected by the simple addition of the effects of the component drugs. Such synergistic interactions between drugs may

improve therapeutic results in cancer treatment if the synergism is specific for tumor cells (Chou and Talalay, 1984). Moreover, analysis of the nature of the interaction between two drugs (synergism, addition, or antagonism) may yield insight into the biochemical mechanisms of interaction of the drugs. For example, two drugs targeting the same enzyme or biochemical pathway may compete with one another resulting in an antagonistic interaction, whereas two drugs targeting completely independent pathways may be additive, and one drug which potentiates the action of another may result in therapeutic synergy.

In order to characterize the effects of combinations of rhuMAb HER2 cytotoxic chemotherapeutic drugs commonly used in breast cancer therapy, we utilized the median-effect/combination-index isobologram method of multiple drug effect analysis. With this methodology, combination index (CI) values are calculated for different dose-effect levels based on parameters derived from median-effect plots of the chemotherapeutic drugs alone, rhuMAb HER2 alone, and the combination of the two at fixed molar ratios. CI values <1 indicate synergy, $CI = 1$ indicates addition, and $CI > 1$ denotes antagonism (Chou and Talalay, 1984). We performed this analysis with rhuMAb HER2 in combination with eight drugs representing seven different classes of cytotoxic chemotherapeutics in vitro. Assays were performed in vitro for drug/rhuMAb HER2 combinations at clinically relevant drug/antibody concentrations using a cytotoxicity endpoint employing SK-BR-3 human breast cancer cells which contain HER-2/neu gene amplification/overexpression. In addition, to circumvent the possibility that any observed interaction might be unique to an individual cell line or to a specific method of analysis, parallel studies were conducted in vivo with the same rhuMAb HER2/drug combinations. HER-2/neu-transfected MCF7 human breast carcinoma xenografts which, in contrast to SK-BR-3 cells are tumorigenic in athymic mice, served as the tumor target for the in vivo studies. Using this model we also investigated the effect of various chemotherapeutic drugs on the pharmacokinetics of rhuMAb HER2 in a subset of mice receiving either rhuMAb HER2 alone or rhuMAb HER-2 plus cytotoxic drug. Finally, we

sought to assess the effect of xenograft size (i.e. tumor burden) on rhuMAb HER2 serum concentrations.

Results

Multiple drug effect analysis of rhuMAb HER2 in combination with cytotoxic chemotherapy drugs on SK-BR-3 breast carcinoma cells in vitro

To extend the observations on anti-HER2 monoclonal antibodies in combination with CDDP, and to conduct a comprehensive survey of rhuMAb HER2 in combination with other classes of cytotoxic chemotherapeutic drugs available for clinical use, rhuMAb HER2 was analysed in combination with seven different drug classes. Representative drugs included: the anthracycline antibiotic, doxorubicin (DOX); the taxane drug, paclitaxel (TAX); a topoisomerase II inhibitor etoposide (VP-16); a platinum analog cisplatin (CDDP); a vinca alkaloid vinblastine (VBL); the alkylating agents, thiotepa (TSPA) for in vitro experiments and cyclophosphamide (CPA) for in vivo experiments; and the antimetabolite drugs methotrexate (MTX) and 5-fluorouracil (5-FU).

In this analysis, dose response curves were constructed for each drug alone, rhuMAb HER2 alone, and the combinations at fixed molar ratios defined as the ratio of the two agents at their maximally effective dose. A representative example of the multiple drug effect analyses performed for all of the chemotherapeutic agent/rhuMAb HER2 combinations is shown for the alkylating agent TSPA (Figure 1 and Table 1). In this analysis Fa and Fu are the fractions of SK-BR-3 cells affected or unaffected, respectively, by the dose (D) of either agent (drug or antibody). DM is the dose required to produce the median effect (analogous to the IC_{50}), and m is the Hill coefficient used to determine whether the dose effect relationships follow sigmoidal dose-response curves (Hill, 1913). Linear regression correlation coefficients (r-values) of the median effect plots (Table 1) reflect that the dose-effect relationships for TSPA, rhuMAb HER2, and the combination, con-
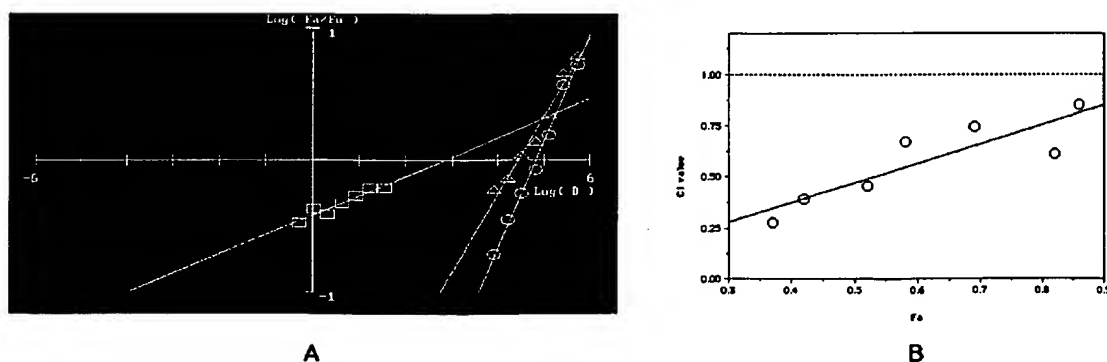

A   B

Figure 1 (a) Multiple drug effect plot of TSPA, rhuMAb HER2 and the combination where Fa = the fraction of SK-BR-3 cells affected by the drugs, Fu = the fraction of cells unaffected, and D = drug dose. (b) Combination Index values for TSPA in combination with rhuMAb HER2 at multiple effect levels. CI values <1 indicate synergy

form to the principle of mass action (in general, r-values >0.9 confirm the validity of this methodology) (Chou and Talalay, 1984). CI values for the combination of TSPA and rhuMAb HER2 were significantly less than 1.0 across all combination doses tested ($P = 0.0008$) indicating a synergistic interaction (Figure 1b). A summary of the data from the same analysis applied to each of the eight cytotoxic drug/rhuMAb HER2 combinations tested (Table 2) demonstrates that CDDP, TSPA, and VP-16 exhibit synergistic therapeutic interactions (CI < 1; $P < 0.001$) with rhuMAb HER2 across a wide range (~0.2–0.8) of Fa values. Additive interactions (CI = 1) were observed for TAX, DOX, MTX, and VBL in combination with rhuMAb HER2, while only one drug, 5-FU, was found to exhibit an antagonistic (CI > 1; $P = 0.0001$) interaction (Table 2).

P185$^{HER-2/neu}$ expression and tyrosine phosphorylation following exposure to cytotoxic agents

Previous work has demonstrated that exposure of several cancer cell lines to the anthracycline DOX results in an increase in expression of the EGFR and/or its ligand TGF-α (Zuckier and Tritton, 1983; Hanauske et al., 1987; Baselga et al., 1992, 1993). This phenomenon has been proposed to explain the synergistic cytotoxic effects of DOX used in combination with anti-EGFR monoclonal antibodies (Baselga et al., 1992). To test whether p185$^{HER-2/neu}$ expression is similarly altered by DOX, protein expression levels were measured at various times following DOX exposure (Figure 2a). These studies demonstrate that following exposure to DOX, p185$^{HER-2/neu}$ expression levels in SK-BR-3 breast carcinoma cells are unaltered, unlike the reported effects of DOX on EGFR expression in A431 cells (Baselga et al., 1992). We next considered the possibility that cytotoxic drugs may impact p185$^{HER-2/neu}$ functional activity rather than expression levels. We therefore determined the effect of the various cytotoxic drugs on heregulin B-1 and 4D5-induced tyrosine phosphorylation of p185$^{HER-2/neu}$
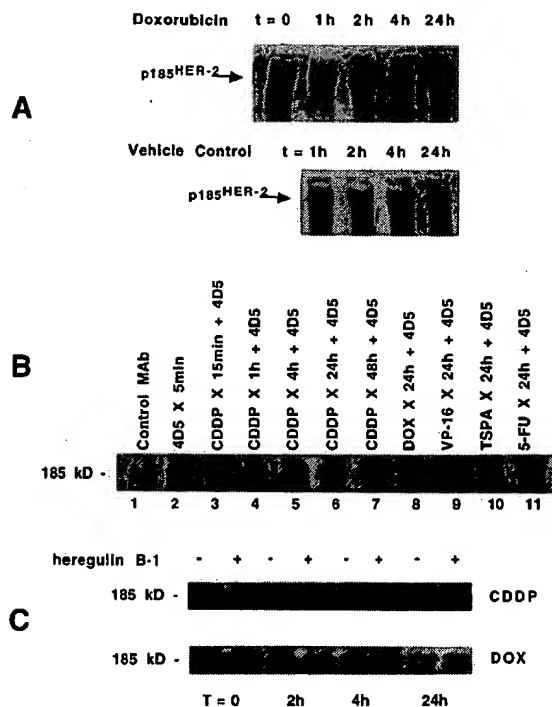

Figure 2 (a) Expression of p185$^{HER-2/neu}$ in SK-BR-3 cells following exposure to DOX at the IC_{30} (30 nM) concentration for the times indicated. (b) MAb 4D5-induced tyrosine phosphorylation of p185$^{HER-2/neu}$ in SK-BR-3 cells following exposure to chemotherapeutic agents at the IC_{30} concentration at the indicated time points. 4D5-associated tyrosine phosphorylation (lane 2) was observed under all of the chemotherapy conditions tested (lanes 3–11) compared to control (lane 1). (c) Heregulin-induced p185$^{HER-2/neu}$ tyrosine phosphorylation in MCF7 cells following exposure to chemotherapeutic drugs at the IC_{30} concentration. These data demonstrate that p185$^{HER-2/neu}$ expression and phosphorylation state are unaltered by prior exposure to the chemotherapeutic agents tested

Table 1 Calculated values for the Combination Index as a function of fractional inhibition of SK-BR-3 cell proliferation by a mixture of TSPA and rhuMAb HER2

| Drug | Combination Index Values | | | | | Parameters | | |
	ED30	ED40	ED50	ED60	ED70	Dm	m	r
TSPA						66.2 μM	0.81	0.99
rhuMAb HER2						675.0 nM	0.15	0.96
TSPA + rhuMAb HER2	0.52	0.37	0.41	0.49	0.60	27.1 μM	0.59	0.99
Diagnosis of combined effect	Synergy	Synergy	Synergy	Synergy	Synergy			

Table 2 Mean combination index values for chemotherapeutic drug/rhuMAb HER2 combinations in vitro

Drug	rhuMAb HER2/drug molar ratio	Drug Dose Range (μM)	Combination Index (Mean ± s.e.m.)	P value	Interaction
TSPA	6.4×10^{-5}	$8.25 – 1.06 \times 10^3$	0.67 ± 0.12	0.0008	Synergy
CDDP	4.0×10^{-4}	$6.5 \times 10^{-1} – 1.7 \times 10^2$	0.56 ± 0.15	0.001	Synergy
VP-16	9.9×10^{-4}	$2.6 \times 10^{-1} – 6.8 \times 10^1$	0.54 ± 0.15	0.0003	Synergy
DOX	9.8×10^{-3}	$2.7 \times 10^{-2} – 6.9$	1.16 ± 0.18	0.13	Addition
TAX	1.4×10^{-1}	$1.8 \times 10^{-3} – 5.0 \times 10^{-1}$	0.91 ± 0.23	0.21	Addition
MTX	3.3×10^{-1}	$8.0 \times 10^{-4} – 2.0 \times 10^{-1}$	1.36 ± 0.17	0.21	Addition
VBL	1.7	$1.6 \times 10^{-4} – 3.9 \times 10^{-2}$	1.09 ± 0.19	0.26	Addition
5-FU	8.8×10^{-5}	$3.0 – 7.65 \times 10^2$	2.87 ± 0.51	0.0001	Antagonism

P values indicate level of significance compared to CI = 1.0

(Yarden, 1990; Holmes *et al.*, 1992). MCF7 or SK-BR-3 breast carcinoma cells were treated with cytotoxic drugs, then allowed to incubate with heregulin (10 nM), or 4D5 (12.5 μg/ml). Protein lysates were then analysed by anti-phosphotyrosine immunoblot. These studies demonstrate an increase in p185[HER-2/*neu*] tyrosine phosphorylation following incubation with 4D5 compared to a non-specific isotype control antibody (Figure 2b, lanes 1 and 2). Prior exposure of the cells to the three drugs which were found to be synergistic with anti-HER-2/*neu* antibody (CDDP, TSPA, and VP-16) had no effect on 4D5-induced p185 tyrosine phosphorylation (Figure 2b, lanes 3–7 and lanes 9 and 10). Similarly, neither DOX which is additive, nor 5-FU which is antagonistic, had effects on 4D5-induced p185 tyrosine phosphorylation (Figure 2b, lanes 8 and 11). In addition, when heregulin B-1 is used to activate p185[HER-2/*neu*] kinase, preincubation of MCF7 breast carcinoma cells with CDDP or DOX had no effect on heregulin-induced p185[HER-2/*neu*] tyrosine phosphorylation (Figure 2c). Preincubation of MCF7 cells with TSPA, VP-16, TAX, MTX, VBL, or 5-FU likewise had no effect on heregulin-induced p185[HER-2/*neu*] tyrosine phosphorylation (data not shown). Taken together

these data demonstrate that none of the synergistic, additive, or antagonistic effects of chemotherapeutic drugs with anti-HER-2/*neu* antibody can be explained on the basis of either chemotherapy-induced alteration of p185[HER-2/*neu*] protein expression levels, or its phosphorylation.

Anti-HER-2/neu *antibodies alter cell cycle distribution of HER-2*/neu-*overexpressing human breast cancer cells*

The cytotoxic effects of antimetabolite drugs are cell cycle dependent (Tannock, 1978). To identify a possible mechanism for the antagonism of 5-FU with rhuMAb HER2 we investigated the effects of murine 4D5 and rhuMAb HER2 on cell cycle distribution of exponentially growing SK-BR-3 and MCF7 cells *in vitro* (Figures 3 and 4). Both the murine 4D5 and rhuMAb HER2 antibodies reduce the percentage of cells undergoing S phase as well as increase the percentage of cells in G0/G1, and these effects are dose-dependent with the maximal antiproliferative activity occurring at antibody concentrations between 1 and 10 μg/ml (Figure 4). There was no significant difference in the magnitude of decrease in S phase
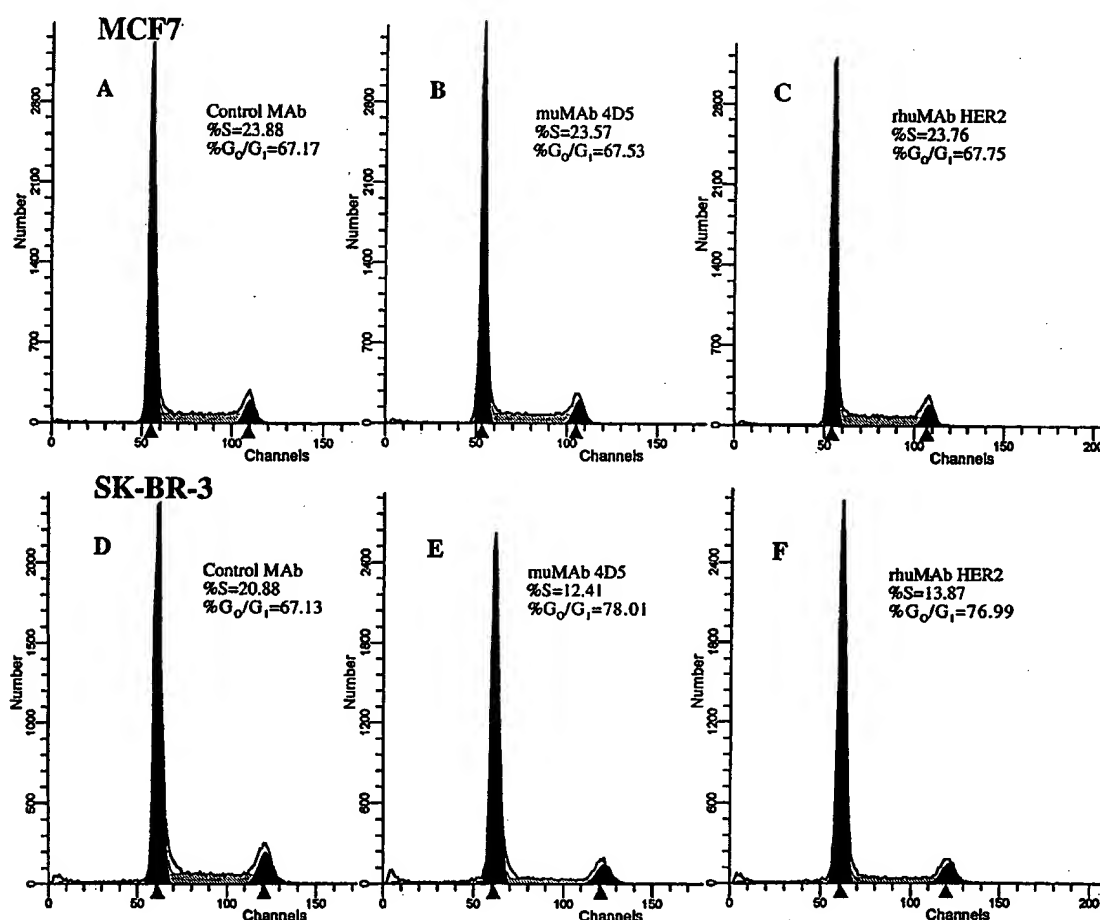

Figure 3 DNA fluorescence flow cytometry histograms of propidium iodide-stained nuclei obtained from MCF7 (a–c) and SK-BR-3 (d–f) breast carcinoma cells following treatment with control antibody 6E10, murine anti-p185[HER-2/*neu*] antibody 4D5, or humanized anti-p185[HER-2/*neu*] antibody (rhuMAb HER2) at a dose of 1 μg/ml for 72 h. These data demonstrate a significant reduction in the fraction of breast carcinoma cells undergoing S phase following treatment with anti-HER-2 antibodies 4D5 and rhuMAb HER2. This effect is specific for cells with HER-2/*neu*-overexpression (SK-BR-3 cells)
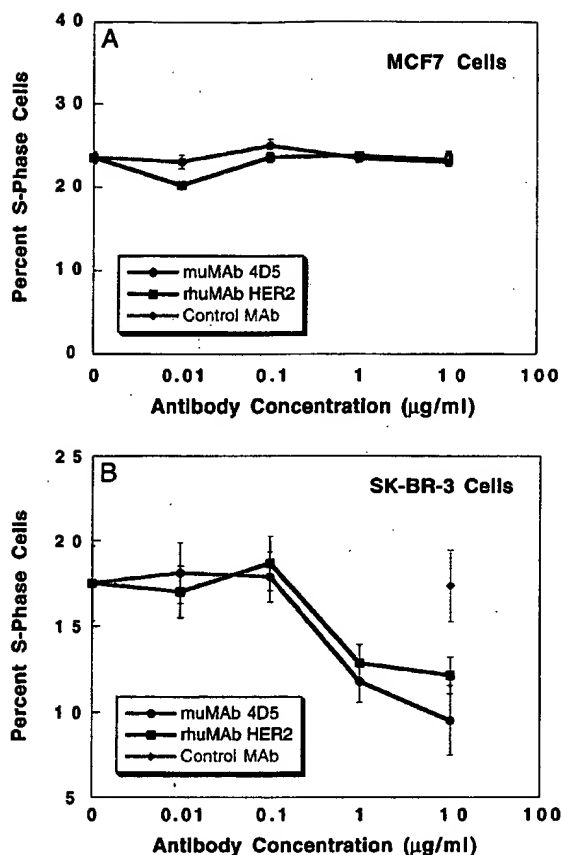
Figure 4 Effect of anti-p185$^{HER-2/neu}$ MAb dose on cell cycle distribution of breast cells without (a) and with (b) HER-2/neu overexpression

fraction of SK-BR-3 cells comparing 4D5 and rhuMAb HER2 indicating the humanization of the murine antibody did not adversely impact its antiproliferative activity. The lack of any effect on cell cycle distribution of MCF7 cells demonstrates the specificity of these antibodies for cells with HER-2/neu overexpression. These data suggest that a decrease in the percentage of SK-BR-3 cells in S phase may result in a decreased sensitivity to 5-FU. An antagonistic interaction for the combination of rhuMAb HER2 with the antimetabolite MTX was not observed. The lack of antagonism between MTX and rhuMAb HER2 in vitro may be due to the longer incubation period required for MTX (120 vs 72 h) to elicit cytotoxicity in the assay used for the multiple drug effect analysis, and the fact that MTX exerts cytotoxic effects in other phases of the cell cycle in addition to S phase (Buick, 1994).

Effect of rhuMAb HER2 in combination with multiple chemotherapeutic drugs on growth of HER-2/neu-transfected MCF7 breast xenografts in vivo

To further evaluate the potential therapeutic effects of rhuMAb HER2/chemotherapy combinations and to extend our observations beyond a single cell line and preclinical model, a series of *in vivo* studies were performed using human breast cancer xenografts in

athymic mice. All of the doses, routes of administration, and dose intervals for the various cytotoxic drugs and rhuMAb HER2 were based on independent dose finding experiments for this specific strain, age, weight, and sex of athymic mouse. The cytotoxic drug doses used were at or near the maximum tolerated doses previously reported in the literature (Giovanella et al., 1977; Boven and Winograd, 1991).

For the alkylating agent cyclophosphamide CPA, combination with rhuMAb HER2 resulted in a significant reduction ($P<0.05$) in day 21 xenograft volume compared to either agent alone (Figure 5a). The combination of the anthracycline antibiotic DOX plus rhuMAb HER2 also significantly reduced MCF7/HER-2 xenograft volume compared to either single agent alone (Figure 5b). The combination of the taxane compound TAX plus rhuMAb HER2, which demonstrated an additive interaction *in vitro*, resulted in a significant reduction in day 20 xenograft volume compared to treatment with TAX alone (Figure 5c). However, the difference between rhuMAb HER2 alone and rhuMAb HER2 plus TAX did not reach statistical significance. This is likely due to the relatively small sample size in each group and the fact that the dose of rhuMAb HER2 in this particular analysis (10 mg/kg I.P. twice weekly) yielded a marked reduction in xenograft growth even when used as a single agent.

The following four rhuMAb HER2/drug combinations were studied in a single *in vivo* experiment. For this experiment, a 'rational dose' (RD) or rhuMAb HER2 was chosen as new information became available based on comparative pharmacokinetic studies from both humans and athymic mice. RD is the dose of a given drug which can reproduce a serum level in experimental animals similar to that observed in human subjects (Inaba et al., 1988). The RD for rhuMAb HER2 resulted in a lower cumulative rhuMAb HER2 dose (16 mg/kg vs 30-50 mg/kg) during the 21 day observation period for this experiment compared to the three *in vivo* studies reported above. With this approach, a significant reduction in day 21 xenograft volume was observed for the topoisomerase II inhibitor VP-16 when used in combination with rhuMAb HER2 compared to either agent alone (Figure 6a). The combination of the microtubule inhibitor VBL with rhuMAb HER2 also significantly reduced MCF7/HER-2 xenograft volume compared to treatment with VBL alone or single agent rhuMAb HER2 (Figure 6b). For the antimetabolite class of cytotoxic chemotherapeutics, two drugs with clinical activity against breast cancer were chosen for combination studies. Treatment with MTX, which targets dihydrofolate reductase, plus rhuMAb HER2 resulted in a significant reduction in day 21 MCF7/HER-2 xenograft volume when compared to either MTX alone or rhuMAb HER2 alone (Figure 6c). Finally, the antimetabolite drug 5-FU, which targets thymidylate synthetase, and which was found to be antagonistic when combined with rhuMAb HER2 *in vitro*, did not yield a significant reduction in xenograft volume when compared to 5-FU alone *in vivo* (Figure 6d). Although the combination of rhuMAb HER2 plus 5-FU was superior to rhuMAb HER2 alone in this experiment ($P<0.05$), the 5-FU dose used had sufficient anti-tumor efficacy as a single agent such
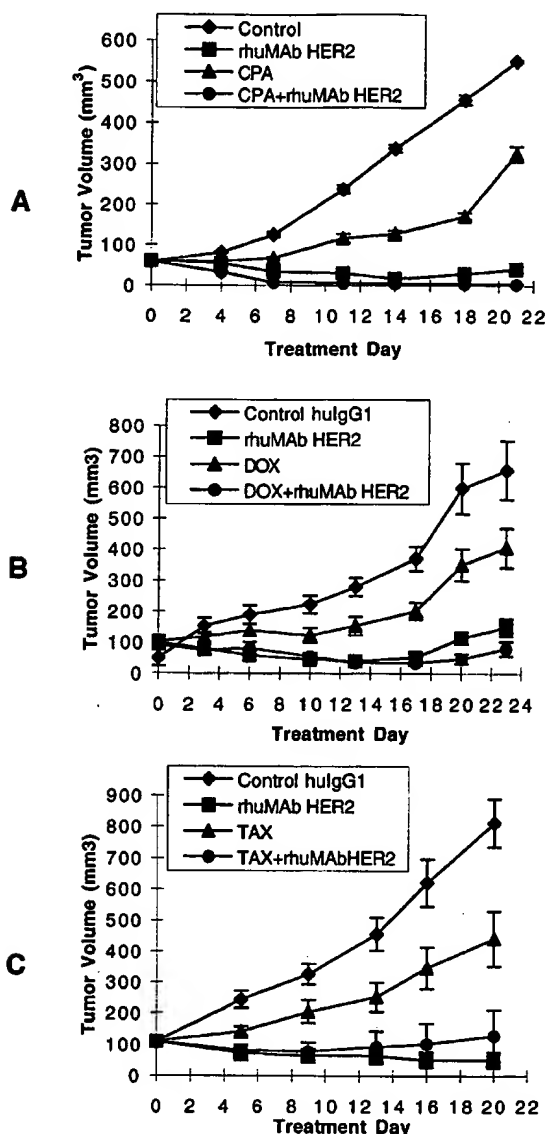
Figure 5 Combination treatment of MCF7/HER-2 breast carcinoma xenografts in athymic mice with rhuMAb HER2 plus chemotherapeutic agents CPA (a), DOX (b), and TAX (c). For each drug, significant reduction in xenograft volume was observed for rhuMAb HER2/drug combinations compared to drug alone controls ($P<0.05$)

that it was not possible to resolve potential differences between 5-FU alone and the combination with the sample sizes chosen (10 mice/group).

Correlation between rhuMAb HER-2 serum concentration and MCF7/HER-2 xenograft volume

To investigate the relationship between rhuMAb HER2 concentration and xenograft size, trough rhuMAb HER2 serum concentration was measured in a subset of mice on day 64 following extended rhuMAb HER2 treatment at the RD (8 mg/kg loading dose and eight weekly i.p. injections of 4 mg/kg) (Figure 7). A significant inverse correlation (Spearman Rank Corre-

lation rho = −0.543; $P = 0.0067$) between trough rhuMAb HER2 concentration and xenograft volume was observed, suggesting that the MCF7/HER-2 xenograft size significantly affects rhuMAb HER2 pharmacology. Furthermore, this effect is independent of serum shed HER-2/*neu* ECD concentration as this molecule was undetectable in any of the murine serum samples analysed (data not shown).

To determine if chemotherapeutic drugs have an effect on rhuMAb HER2 pharmacology, day 64 trough serum rhuMAb HER2 concentrations were analysed by treatment group in a subset of mice used for the *in vivo* studies. Controlling for xenograft size, there was no significant difference in rhuMAb HER2 trough concentration between any of the treatment groups in Figure 7 (data not shown).

Discussion

The protein products of transforming oncogenes have been a target for anti-cancer drug development since the initial discovery of these genes, however there is only one currently approved drug specifically targeting these proteins in clinical use. Identification of the HER-2/*neu* gene alteration and its association with aggressive forms of human breast cancer has resulted in its successful therapeutic targeting (Slamon *et al.*, 1987, 1989; Baselga *et al.*, 1996; Pegram *et al.*, 1998). The interaction of anti-HER-2/*neu* antibodies with p185[HER-2/neu] results in receptor tyrosine phosphorylation. (Yarden, 1990), downregulation of receptor expression (Park *et al.*, 1992), internalization of the antibody-receptor complex (Maier *et al.*, 1991), and a decrease in the association of p185[HER-2/neu] with its heterodimeric partners HER-3 and/or HER-4 (Reese *et al.*, 1996; Klapper *et al.*, 1997). These events are accompanied by a number of biological effects including most importantly a decrease in cell proliferation (Rodriguez *et al.*, 1993), alteration of cell cycle distribution, and a marked decrease in the ability of the cell to excise and repair DNA damage induced by platinum analogs (Pietras *et al.*, 1994; Arteaga *et al.*, 1994). This enhanced cytotoxic activity is specific for malignant cell lines or xenografts with HER-2/*neu* receptor overexpression since anti-HER-2/*neu* antibodies have no such effect on cell lines with physiologic HER-2/*neu* expression levels (Hancock *et al.*, 1991; Pietras *et al.*, 1994). Interaction between the p185[HER-2/neu] signaling pathway and CDDP-DNA repair mechanisms has been confirmed using tyrosine kinase inhibitors to block p185[HER-2/neu] receptor phosphorylation which inhibits antibody induced attenuation of repair of platinum-DNA adducts (Arteaga *et al.*, 1994). Moreover, reversal of CDDP resistance is possible through transfection and over-expression of HER-2/*neu* cDNA followed by incubation with anti-HER-2/*neu* antibody (Pietras *et al.*, 1994). As a result of this work, studies demonstrating the clinical efficacy of the combination of an anti-HER-2/*neu* antibody plus CDDP were conducted in breast cancer patients with HER-2-overexpressing breast cancers who previously exhibited clinical drug resistance to cytotoxic therapy (Pegram *et al.*, 1998).

To test whether this receptor enhanced chemosensitivity mechanism could be observed with other classes
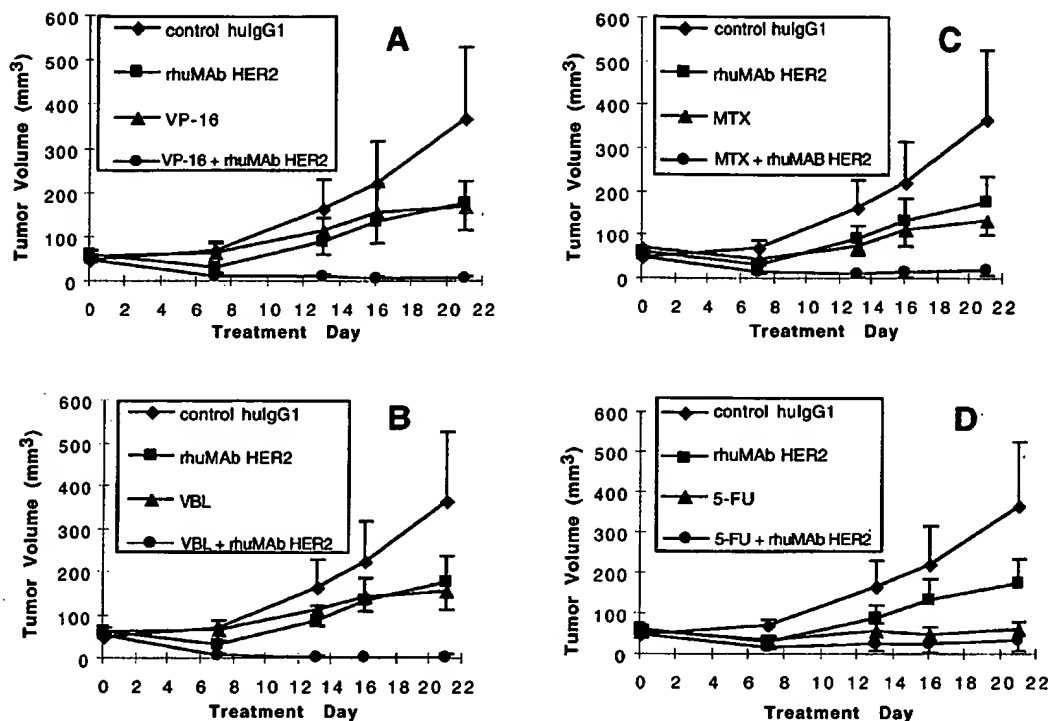
Figure 6 Treatment of MCF7/HER2 xenografts with rhuMAb HER2 in combination with VP-16 (a), VBL (b), MTX (c), and 5-FU (d). Combination drug/rhuMAb HER2 treatment resulted in a significant reduction in xenograft volume compared to drug alone, or rhuMAb HER2 alone, controls ($P<0.05$) for each of the drugs indicated with the exception of 5-FU
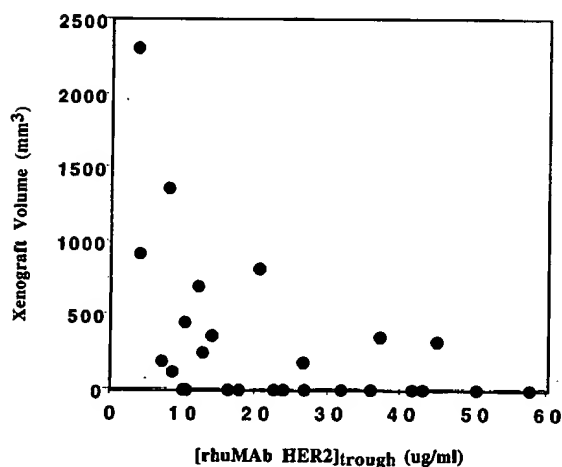

Figure 7 Inverse relationship between MCF7/HER-2 xenograft volume and trough rhuMAb HER2 concentration in murine serum (Spearman Rank Correlation rho = −0.543; $P=0.0067$). These data suggest that binding of rhuMAb HER2 to HER-2/neu-overexpressing xenografts reduces serum rhuMAb HER2 concentrations

of cytotoxic chemotherapeutic agents, we performed a series of studies evaluating combinations of cytotoxic agents with rhuMAb HER2 testing seven classes of chemotherapeutics in common clinical use. All concentration ranges of cytotoxic drugs and rhuMAb HER2 tested in these studies were conducted at serum

concentrations achieved in humans (Pegram et al., 1997, 1998). Data from the multiple drug effect analysis methodology are useful, not only in establishing hypotheses as to the mechanism of action of multidrug combinations, but can also provide insight as to how two drugs should be administered temporally to gain the maximum therapeutic effect. For example, two drugs which are synergistic might best be administered together whereas two antagonistic drugs would be most effective if given sequentially. Data from the current study demonstrate that the platinum compound CDDP, the alkylating agent TSPA, and the topoisomerase II inhibitor VP-16 are synergistic in combination with rhuMAb HER2 in treating HER-2/neu-overexpressing SK-BR-3 breast carcinoma cells in vitro. These results suggest the possibility of an interaction between the HER-2/neu signaling pathway and intracellular DNA repair mechanisms involved with repair of DNA damage resulting from these specific DNA damaging agents. Other potential mechanisms might also explain the synergy observed between rhuMAb HER2 and these agents, including the possibility that rhuMAb HER2 could impact the cellular pharmacology of the drugs resulting in an increase in their cytotoxic activity. An argument against this hypothesis is the fact that the anti-HER-2/neu antibody has no effect on the net cellular incorporation of ^{14}C-labeled carboplatin (Pietras et al., 1994) or [14C]-doxorubicin in target cells (Pegram et al., 1992). Another possible mechanism for the observed synergy with rhuMAb HER2 is an effect of cytotoxic drugs on the expression level and/or kinase

activity of p185$^{HER-2/neu}$. An analogous mechanism has been postulated for the EGFR where low doses of DOX appear to increase receptor expression enhancing the antiproliferative activity of anti-EGFR antibody (Zuckier and Tritton, 1983; Hanauske et al., 1987; Baselga et al., 1992, 1993). The current data demonstrate no change in p185$^{HER-2/neu}$ expression levels or in HER-2/neu receptor tyrosine phosphorylation following exposure to cytotoxic drugs, suggesting that unlike the EGFR, this mechanism is not operative for the HER-2/neu receptor.

Most of the rhuMAb HER2/drug combinations evaluated in this study demonstrate additive rather than synergistic interactions suggesting that the majority of observed antiproliferative effects of rhuMAb HER2 plus cytotoxic drugs are due to a mechanism of action involving each agent acting independently. It is interesting to note that the mechanisms of action of many of the drugs demonstrating additivity do not involve direct DNA damage, but rather disruption of microtubule polymerization/depolymerization (taxanes and vinca alkaloids) or inhibition of DNA synthesis (antimetabolites). This observation is consistent with the hypothesis that the synergy between cytotoxic drugs and rhuMAb HER2 involves an interaction between the HER-2/neu signaling and DNA repair pathways. Subsequent to our initial demonstration of the additive effects of rhuMAb HER2 with TAX (Hsu et al., 1997), studies confirming this additive interaction were published (Baselga et al., 1998). The antimetabolite drug 5-FU is the only drug which demonstrated antagonism when used in combination with rhuMAb HER2 in vitro. We have not yet defined the mechanism of this interaction, but it may be the result of alterations in cell cycle distribution caused by rhuMAb HER2 as seen in the current data. It could also be the result of intracellular pharmacological effects, alteration of the enzymatic activity responsible for conversion of 5-FU to 5-fluorodeoxyuridine monophosphate, or an impact on the level of the target enzyme thymidylate synthetase. Further work is needed to explore these possibilities.

The multiple drug effect model is not easily applied to analysis of in vivo studies since such analyses, with the number of drugs reported in this study, would require at least 600 athymic mice (assuming five mice per group, five data points for each dose response curve, and three dose response curves – for each drug alone, and in combination with rhuMAb HER2). Consequently we used a more conventional approach for analysis of the in vivo data (i.e. single factor ANOVA at fixed time points following treatment of mice with optimal drug or rhuMAb HER2 doses). The cytotoxic drug doses chosen for these experiments are at or near the MTD reported in the literature for each of the cytotoxic drugs. The rhuMAb HER2 doses and schedules were designed to achieve target serum concentrations of $\geqslant 10-20$ μg/ml in mice bearing HER-2/neu-overexpressing xenografts of 50–500 mm^3 in size. This antibody concentration is associated with our previously published maximal antiproliferative effect in vitro (De Santes et al., 1992). With this in vivo approach, we demonstrated significantly superior anti-tumor efficacy of rhuMAb HER2 in combination with CPA, DOX, MTX, TAX, VP-16, and VBL when compared to effects of each chemotherapeutic drug

alone. These results are consistent with the in vitro data which demonstrate that rhuMAb HER2 is either additive or synergistic with each of these drugs. For the drug 5-FU, which was antagonistic with rhuMAb HER2 in vitro, the same combination in vivo was superior to rhuMAb HER2 alone but not to 5-FU alone. Although this could be secondary to an antagonistic effect, it is also possible that the sample sizes in each treatment group were not sufficient to discriminate between 5-FU alone and the combination, especially in light of the fact that single agent 5-FU had a marked effect on xenograft volume in this model. It is important to note that in the analysis of the combination studies in vivo, rhuMAb HER2 had no deleterious effect on chemotherapeutic drug efficacy. Additionally, we did not observe any overt increase in toxicity, as determined by measurement of animal weights, observations of activity level, and overall survival, in mice treated with rhuMAb HER2/chemotherapy combinations.

Previous analysis of rhuMAb HER2 pharmacokinetics in human subjects demonstrate an inverse association between serum concentrations of rhuMAb HER2 and the shed HER-2/neu ECD (Pegram et al., 1998). One mechanism which may explain this observation is the direct binding of rhuMAb HER2 to shed HER-2/neu ECD in the circulation resulting in a more rapid clearance of the resulting antigen/antibody complex by the reticuloendothelial system. Another potential mechanism is that high serum shed HER-2/neu ECD may be a marker of increased tumor burden, resulting in an inverse association between rhuMAb HER2 concentration and shed HER-2/neu ECD due to increased binding and turnover of rhuMAb HER2 directly by tumor cells. In the MCF7/HER-2 xenograft model, we measured rhuMAb HER2 trough concentration, shed HER-2/neu ECD, and tumor volume concurrently. These data demonstrate a significant inverse relationship between rhuMAb HER2 trough concentration and xenograft volume. This relationship is independent of serum shed HER-2/neu ECD since no serum shed HER-2/neu ECD could be detected using a sensitive ELISA assay (Sias et al., 1990) in this model. These data demonstrate that tumor burden alone in the absence of shed HER-2/neu ECD is sufficient to affect rhuMAb HER2 pharmacokinetics. In addition the current data demonstrate that prior treatment with the drugs MTX, 5-FU, VP-16, and VBL in vivo had no effect on rhuMAb HER2 trough levels in murine serum. Consistent with this is the published data showing concomitant administration of the drug CDDP had no impact on mean pharmacokinetic parameters of rhuMAb HER2 in a phase II clinical trial of CDDP plus rhuMAb HER2 in 39 patients with advanced breast cancer (Pegram et al., 1998). Taken together, these data suggest that the cytotoxic chemotherapeutic drugs evaluated have no effect on rhuMAb HER2 pharmacokinetics in vivo.

It is now generally accepted that identification of molecular alterations which play a role in the pathogenesis of specific human malignancies will lead to the development of targeted therapeutics which should be more effective and less toxic than currently available agents. Activation of HER-2/neu resulting from gene amplification in human breast cancer is one of what is hoped to be a number of molecular targets

for future drug design in this disease as well as other human cancers. Studies leading to a greater understanding of the biological consequences of HER-2/neu-directed therapies should allow the integration of this molecularly-targeted approach with currently available cancer treatments. The additive or synergistic therapeutic interaction between rhuMAb HER2 and a number of chemotherapeutic drugs suggests that such combinations could be successfully exploited in future human clinical trials.

Materials and methods

Multiple drug effect analysis of rhuMAb HER2 in combination with cytotoxic chemotherapeutic agents against HER-2/neu-overexpressing SK-BR-3 breast carcinoma cells in vitro

Aliquots of 5×10^3 SK-BR-3 cells were plated in 96-well microdilution plates. Following cell adherence (24 h), experimental media containing either rhuMAb HER2 (Genentech, Inc. South San Francisco, CA, USA) or control media was added to appropriate wells. After incubation for 24 h, chemotherapeutic agent or control solution was added to triplicate wells and serial twofold dilutions were performed to span the dose range ($\sim EC_{10}-EC_{90}$) suitable for the dose-effect analysis for rhuMAb HER2 and each of the cytotoxic drugs. The dose ranges for rhuMAb HER2 and each drug tested in these experiments are listed in Table 2. We have previously shown that these doses are relevant to drug/rhuMAb HER2 concentrations achievable in human subjects (Pegram et al., 1997, 1998). Eight cytotoxic drugs representative of seven different classes of cytotoxic chemotherapeutic agents were analysed including: platinum analogs – cisplatin (CDDP; Bristol Laboratories, Princeton, NJ, USA); anthracycline antibiotics – doxorubicin (DOX; Cetus Corporation, Emeryville, CA, USA); alkylating agents – thiotepa (TSPA; Lederle Laboratories, Pearl River, NY, USA); taxanes – paclitaxel (TAX; Mead Johnson, Princeton, NJ, USA); vinca alkaloids – vinblastine (VBL; Eli Lilly Co., Indianapolis, IN, USA); topoisomerase II inhibitors – etoposide (VP-16; Bristol Laboratories, Princeton, NJ, USA); and antimetabolites – 5-fluorouracil (5-FU; Solo Park Laboratories, Inc., Elk Grove Village, IL, USA) and methotrexate (MTX; Immunex Corporation, Seattle, WA, USA).

Following incubation for 72 h (120 h for MTX) plates were washed with PBS and stained with 0.5% N-Hexamethylpararosaniline (crystal violet) in methanol. Sorenson's buffer (0.025 M sodium citrate, 0.025 M citric acid in 50% ethanol) 0.1 ml was added to each well, and the plates were analysed in an ELISA plate reader at 540 nm wavelength. Absorbance at this wavelength correlates with cell survival (Flick and Gifford, 1984; Gillies et al., 1986; Reile et al., 1990). Absorbance values from control wells in each plate were compared statistically to ensure even loading of cells from plate to plate for each experiment. Multiple drug effect analysis was performed using computer software (Biosoft, Cambridge, UK). Details of this methodology have been published previously (Chou and Talalay, 1984; Bible and Kaufmann, 1997). Briefly, the $\log[(1/f) - 1]$ was plotted against log (drug dose) (Figure 1). From the resulting median effect lines, the X-intercept (log EC_{50}) and slope m were calculated for each drug. These parameters were then used to calculate doses of the component drugs (and combinations) required to produce various cytotoxicity levels according to equation (a). For each level of cytotoxicity, combination index (CI) values were then calculated according to equation (b) where $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce survival f, $(Df)_1$ and $(Df)_2$

are the concentrations of the component drugs required to produce f.

$$\text{Dose}_1 = \text{Dose IC}_{50}[(1 - f)/f]^{1/m} \qquad (a)$$

$$CI = (D)_1/(Df)_1 + (D)_2/(Df)_2 + \alpha(D)_1(D)_2/(Df)_1(Df)_2 \quad (b)$$

The CIs were calculated based on the conservative assumption of mutually nonexclusive drug interactions ($\alpha = 1$), i.e. cytotoxic drugs have mechanisms of action unique from rhuMAb HER2. Statistical tests were then applied (student t-test) to determine if the mean CI values resulting from separate experiments at multiple effect levels were significantly different from CI = 1.

Western blot analysis

MCF7 and SK-BR-3 cells were allowed to incubate with cytotoxic drugs at the IC_{30} concentration for the times indicated in Figure 2. Following drug exposure, cells were allowed to incubate with monoclonal anti-HER-2 antibody 4D5 (12.5 μg/ml) for 5 min at 37°C or recombinant heregulin B-1 (10 nM) for 15 min at 37°C or control solutions. Cells were then washed in PBS and lysed at 4°C in 20 mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethyl-sulfonylfluoride, leupeptin 1 μg/ml and aprotinin 1 μg/ml. Insoluble material was cleared by centrifugation and protein was quantitated using BCA (Pierce Biochemicals, Rockford, IL, USA), resolved by SDS–PAGE, and transferred to immobilon-P (Millipore, Bedford, MA, USA). P185[HER-2/neu] protein expression was detected using anti-c-neu (Oncogene Science, Uniondale, NY, USA); and anti-phosphotyrosine immunoblotting was performed using monoclonal antibody PY20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell cycle analysis

SK-BR-3 or MCF7 breast cancer cells were plated at a density of 2×10^6/dish in 60×15-mm culture dishes and allowed to adhere overnight. Monolayers were washed with PBS and allowed to incubate with media containing anti-HER-2 or control antibodies at concentrations of 0.01–10 μg/ml. Following 72 h incubation, cells were trypsinized, washed with PBS, fixed in ice-cold methanol, and stored at −20°C. Fixed cells were then washed twice with PBS and allowed to incubate with RNAse 100 μg/ml (Worthington Biochemical) for 30 min at 37°C. Following centrifugation, nuclei were subjected to propidium iodide 50 μg/ml (Molecular Probes, Inc.) staining in PBS. Samples were analysed by flow cytometry (Epics Elite, Coulter Corporation) using Modfit LT software (Verity Software House).

Analysis of rhuMAb HER2 in combination with cytotoxic chemotherapeutic drugs against HER-2/neu-overexpressing breast carcinoma xenografts in vivo

HER-2/neu-transfected MCF7 cells which express high levels of p185[HER-2/neu] and form xenografts in athymic mice were injected subcutaneously (s.q.) at $\sim 1.0 \times 10^7$ cells/tumor in the mid-back region of 4–6-week-old, female, CD-1 (nu/nu), athymic mice (Charles River Laboratories, Wilmington, MA, USA). Prior to cell injection, all mice were primed with 17β-estradiol (Innovative Research of America, Sarasota, FL, USA) applied s.q. (1.7 mg estradiol/pellet) to promote tumor growth. Tumor volumes, calculated as the product of length, width, and depth, were monitored twice weekly by serial micrometer measurements by a single observer. Five to ten animals were randomly assigned to each treatment group.

Statistical tests were performed (single-factor ANOVA) to assure uniformity in starting tumor volumes between treatment and control groups at the beginning of each experiment. All drugs, with the exception of VP-16 which was administered s.q., were administered by intraperitoneal (i.p.) injection. The dosage of chemotherapeutic agents tested were as follows: DOX (5 mg/kg, day 1), MTX (2 mg/kg, days 1–5), VP-16 (20 mg/kg, days 1–3), 5-FU (16 mg/kg, days 1–4), VBL (0.8 mg/kg, days 1 and 2), cyclophosphamide (CPA; 80 mg/kg, days 0, 4 and 8) and TAX (15 mg/kg, days 1–3). These doses were based on independent dose-finding experiments conducted in our laboratory and were near the maximum-tolerated dose for this specific age and strain of female athymic mice. To assure accurate dosing, drug doses were individualized based upon animal weights determined immediately prior to each injection. Treatment with control antibody, cytotoxic drug, rhuMAb HER2, or the combination was initiated 9–14 days status post xenograft inoculation at which time xenograft volumes measured $\sim$50–100 mm³. Differences in day 21 xenograft volumes between groups were analysed by single-factor ANOVA of the log transformed tumor volume data. Three dosing schedules of rhuMAb HER2 were used for these experiments. All dosing schedules were designed to achieve target serum concentrations of $\geqslant$10–20 µg/ml during the time chemotherapeutics agents were administered. For the *in vivo* experiments with MTX, VP-16, 5-FU, and VBL, the loading dose of rhuMAb HER2 was 8 mg/kg, and the weekly maintenance dose was 4 mg/kg. For the experiments with DOX and CPA, the dose of rhuMAb HER2 was 10 mg/kg, days 0, 4, and 8. And for the *in vivo* experiment with TAX, the rhuMAb HER2 dose was 10 mg/kg twice per week. Human myeloma IgG₁ (Calbiochem-Novabiochem, La Jolla, CA, USA) served as the control antibody for these experiments and was administered at the same dose and dose interval as rhuMAb HER2.

Measurement of rhuMAb HER2 in murine serum

The trough concentration of rhuMAb HER2 in mouse serum was measured using an ELISA with the extracellular domain (ECD) of p185^HER-2/neu as the coat antigen. In this format, 100 µl of p185^HER-2/neu (Genentech, Inc.) was added to MaxiSorp 96-well microtiter plates (Nunc, Roskilde,

Denmark) at 1 mg/ml in 0.05 M sodium carbonate, pH 9.6. After overnight incubation at 2–8°C, plates were washed three times with ELISA wash buffer (PBS containing 0.05% Tween-20) using a Biotek EL304 platewasher (Biotek Instruments, Inc., Winooski, VT, USA). Plates were then blocked with 200 µl/well of ELISA diluent (PBS containing 0.5% BSA, 0.05% Tween-20, and 0.05% Proclin300, pH 7.2) for 1–2 h at ambient temperature with agitation. After blocking, plates were washed again three times with ELISA wash buffer. Subsequently, 100 µl of standards, samples, or controls were added to duplicate wells and allowed to incubate for 1 h at ambient temperature. After incubation, the plates were washed six times in ELISA wash buffer, and 100 µl of PBS, pH 7.2, containing 2.2 mmol orthophenylene diamine (OPD), (Sigma Chemical Co.) and 0.012% (vol/vol) hydrogen peroxide (H₂O₂; Sigma Chemical Co.) were added to each well. When color had fully developed, the reaction was quenched with 100 µl/well of 4.5 molar sulfuric acid. Absorbence values at 492 nm minus 405 nm reference absorbence were measured using an automatic plate reader (Molecular Devices, Palo Alto, CA, USA). A 4-parameter curve fit program was used to generate the standard curve, from which sample and control concentrations were interpolated (SOFTmax). The standard curve range for the assay was 1.56–100 mg/ml.

Detection of p185^HER-2/neu extracellular domain in murine serum

The method for detection of shed HER-2/neu extracellular domain (ECD) levels in serum has been described in detail elsewhere (Sias *et al.*, 1990). Briefly, the ELISA employs pairs of anti-HER-2/neu monoclonal antibodies (Genentech, Inc.) which recognize mutually exclusive determinants of the extracellular domain of p185^HER-2/neu. Wells were coated overnight at 4°C with MAb 7F3 which does not compete with rhuMAb HER2 ECD binding. Assay standards (recombinant, p185^HER-2/neu ECD) and murine serum samples were added to appropriate wells and allowed to incubate for 2 h. Following a wash step, secondary antibody was added (MAb 4D5 to detect free shed HER-2 ECD, and MAb 2C4 to detect total shed HER-2 ECD) for 2 h. The bound conjugate is detected with OPD substrate and the resulting absorbence is measured at 490 nm wavelength. The range of the assay is 8.3–1800 ng/ml.

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Trastuzumab and Chemotherapeutics: Drug Interactions and Synergies

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Previous studies have shown a synergistic interaction between trastuzumab (Herceptin; Genentech, Inc, South San Francisco, CA) and the cytotoxic drug cisplatin in human breast cancer cells. To define the nature of the interaction between trastuzumab and other classes of cytotoxic drugs, we applied multiple drug effect/combination index isobologram analysis to a variety of chemotherapeutic drug/trastuzumab combinations in vitro. Synergistic interactions at clinically relevant drug concentrations were observed for trastuzumab in combination with cisplatin, docetaxel, thiotepa, 4-OH cyclophosphamide, vinorelbine, and etoposide. Additive cytotoxic effects were observed with trastuzumab plus doxorubicin, paclitaxel, methotrexate, and vinblastine. One drug, 5-fluorouracil was found to be antagonistic with trastuzumab in vitro. In vivo drug/trastuzumab studies were conducted with HER-2/neu–transfected MCF7 human breast cancer xenografts in athymic mice. Combinations of trastuzumab plus cisplatin, docetaxel, cyclophosphamide, doxorubicin, paclitaxel, methotrexate, etoposide, and vinblastine in vivo resulted in a significant reduction in xenograft volume compared to chemotherapy-alone controls ($P < .05$). The synergistic interaction of trastuzumab with specific chemotherapeutic agents suggests rational combinations for testing in human clinical trials.
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THE CONCEPT of combining trastuzumab (Herceptin; Genentech, Inc, South San Francisco, CA) with chemotherapy is an old one, dating to the earliest stages of clinical development of this drug. In fact, one of the very first phase I clinical trials of trastuzumab involved study of the combination of trastuzumab with cisplatin. The very first objective clinical responses to trastuzumab were observed in this important trial, including a complete clinical remission in a patient with metastatic breast cancer that is durable to this day, more than 8 years later. The combination of trastuzumab with cisplatin, a drug not frequently used in breast cancer therapy, was based on the observation that HER-2–overexpressing breast cancer cells exposed to trastuzumab have a decrease in the rate of DNA repair, thus rendering cells more sensitive to DNA-damaging agents such as the platinum salts, alkylating agents, and ionizing radiation.[1,2] This effect is specific for HER-2–overexpressing cells and can be blocked by drugs that inhibit HER-2 kinase.[3] A similar phenomenon was first observed with antibodies directed against the closely related human epidermal growth factor receptor, HER-1.[4] This phenomenon, termed receptor-enhanced chemosensitivity, yielded an order of magnitude increase in cell kill with combinations of trastuzumab and DNA-reactive agents, and in computational algorithms that test drug-drug interactions showed synergy between trastuzumab and cisplatin.[1] These early laboratory studies set the stage for subsequent clinical trials of trastuzumab and the platinum salts; this combination showed significant activity without increased toxicity against chemoresistant, advanced breast cancers.[5] Further clinical trials testing the hypothesis of synergy between platinum salts and trastuzumab are planned, including large-scale, prospective, randomized trials for patients with HER-2–overexpressing breast cancers. Support for such clinical trials are critical to maximize efficacy, while at the same time ensuring the safety of trastuzumab in combination with existing chemotherapeutics.

METHODS FOR TESTING TRASTUZUMAB/ CHEMOTHERAPY INTERACTIONS

Our strategy for evaluating the activity and efficacy of new drug combinations using trastuzumab has been to develop preclinical models that can quantitatively measure the interaction between trastuzumab and any drug of interest, particularly those with known clinical activity in breast can-

cer. To this end, we have used the model of the median-effect principle derived by Chou and Talalay[6] to study multiple drug interactions with trastuzumab in cell-based in vitro growth assays. The advantages of this technique are: (1) the model is based on the fundamental laws of mass action, (2) this methodology is one of the most widely accepted and most frequently cited in the medical literature, (3) the results from multiple drug-effect analyses have the potential to be clinically meaningful if the concentration ranges of the study drugs are clinically achievable, and (4) the model provides a quantitative definition of the nature of interaction between trastuzumab and other drugs. These interactions can be classified as either additive, synergistic, or antagonistic.[6] The rationale for such thorough preclinical testing before launching a major clinical effort evaluating a novel drug combination includes the ability to screen a relatively large number of drug interactions (seeking those that are synergistic as the most promising for clinical development), the ability to test schedule dependence of drug combinations, and the ability to prioritize clinical investigations by avoiding studies of combinations that are antagonistic.

A frequent criticism of in vitro drug interaction studies is that the results may not translate into the in vivo setting because in vitro cell proliferation assays cannot recapitulate the complex tumor microenvironment and the dynamic changes in drug concentrations and drug metabolism resulting from the pharmacokinetic profile of the drugs under study. To address this criticism, we have developed a novel in vivo approach (the Lopez model) for evaluating the efficacy of combination antitumor agent dosing schedules that accounts for both the order and timing of drug administration.[7] Our approach compares in vivo tumor volume trajectories over time and offers a quantitative definition for additivity of drug effects, relative to which synergism and antagonism may be classified. The model begins by fitting data from individual mice receiving at most one drug to a differential equation tumor growth/drug-effect model, and combining individual parameter estimates to obtain population statistics. Using the null hypothesis that combination therapy is consistent with additivity, or that combination therapy is equivalent to treating with the more effective single agent alone, we compare predicted tumor growth trajectories and their distribution for combination-

treated animals.[7] The assumptions and definitions underlying this model-based approach may allow for clearer quantification and interpretation of the complex concepts of synergism, additivity, and antagonism for in vivo tumor models, and we hope will hasten the development of rational antitumor drug combinations for clinical testing.

MULTIPLE DRUG-EFFECT ANALYSIS OF TRASTUZUMAB IN COMBINATION WITH CYTOTOXIC CHEMOTHERAPEUTICS AGAINST HER-2–OVEREXPRESSING BREAST CANCER CELLS

We have conducted a comprehensive analysis of trastuzumab combinations with various classes of cytotoxic chemotherapeutics.[8,9] Representative agents from each drug class were chosen (Table 1). In this analysis, dose-response curves were constructed for each drug alone (at clinically achievable doses), trastuzumab alone, and the combinations at fixed molar ratios. Multiple drug-effect analysis was then performed for each trastuzumab/drug combination. A summary of the data from this analysis applied to each of eleven drugs representing seven different classes of chemotherapeutic agents is shown in Table 1. Synergistic interactions were observed between trastuzumab and cisplatin, carboplatin, thiotepa, 4-OH cyclophosphamide, etoposide, vinorelbine, and docetaxel. Additive interactions with trastuzumab were observed for doxorubicin, paclitaxel, methotrexate, and vinblastine. Only one drug, 5-fluoro-

| Table 1. Interaction Between Trastuzumab and Cytotoxic Agents With Activity in Breast Cancer	
Drug	Interaction with Trastuzumab
Cisplatin/Carboplatin	Synergistic
Docetaxel	Synergistic
Vinorelbine	Synergistic
Etoposide	Synergistic
Thiotepa	Synergistic
Ionizing radiation	Synergistic
Doxorubicin	Additive
Vinblastine	Additive
Paclitaxel	Additive
Methotrexate	Additive
5-Fluorouracil	Antagonistic
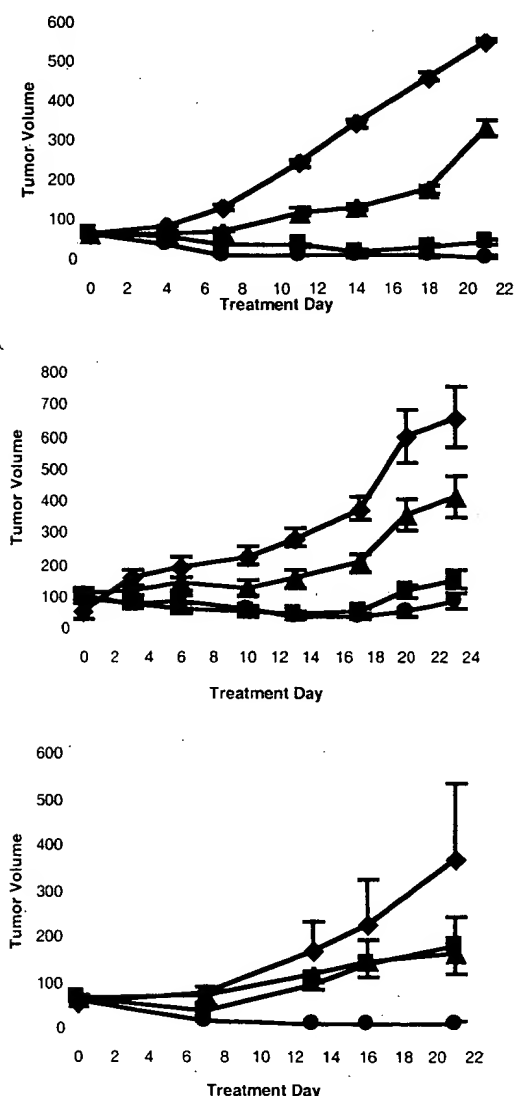
Fig I. Combination treatment of MCF7/HER-2 breast carcinoma xenografts in athymic mice with trastuzumab plus chemotherapeutic agents. Examples shown are cyclophosphamide (top), doxorubicin (center), and vinblastine (bottom). Diamond, control; square, rhuMAb HER-2; triangle, agent; circle, agent + rhuMAb HER-2.

Table 2. Drug/Trastuzumab Compinations That Yield Reductions in Xenograft Volume
Drug
Cisplatin
Docetaxel
Cyclophosphamide
Doxorubicin
Paclitaxel
Methotrexate
Etoposide
Vinblastine

vitro for the various drug combinations. Representative examples from these experiments and a summary of the in vivo data are shown in Figure 1 and Table 2.[8]

A MODEL-BASED APPROACH FOR ASSESSING IN VIVO COMBINATION THERAPY INTERACTIONS

We illustrate this approach by comparing entire observed and expected tumor volume trajectories for a data set in which HER-2–overexpressing MCF7 human breast cancer xenografts were treated with trastuzumab, doxorubicin, or one of five proposed combination therapy schedules.[7]

uracil, was found to be antagonistic with trastuzumab.[8,9]

To further evaluate the potential therapeutic effects of trastuzumab/chemotherapy combinations and to extend our observations beyond cell-based assays, a series of in vivo studies were performed using established human breast cancer xenografts grown in athymic mice.[8] We found that these results closely paralleled the results observed in

Table 3. Preclinical Experimental Design for Chemotherapy/Trastuzumab Dosing Schedules

Treatment Group	Length of Trastuzumab Administration (days)	Length of Doxorubicin Administration (days)
Control	–	–
T, day 0	0	–
T, day I	I	–
T, day 4	4	–
D, day 0	–	0
D, day I	–	I
D, day 4	–	4
T+D, simultaneous administration	0	0
T, day 0; D, day I	0	I
T, day 0; D, day 4	0	4
T, day I; D, day 0	I	0
T, day 4; D, day 0	4	0

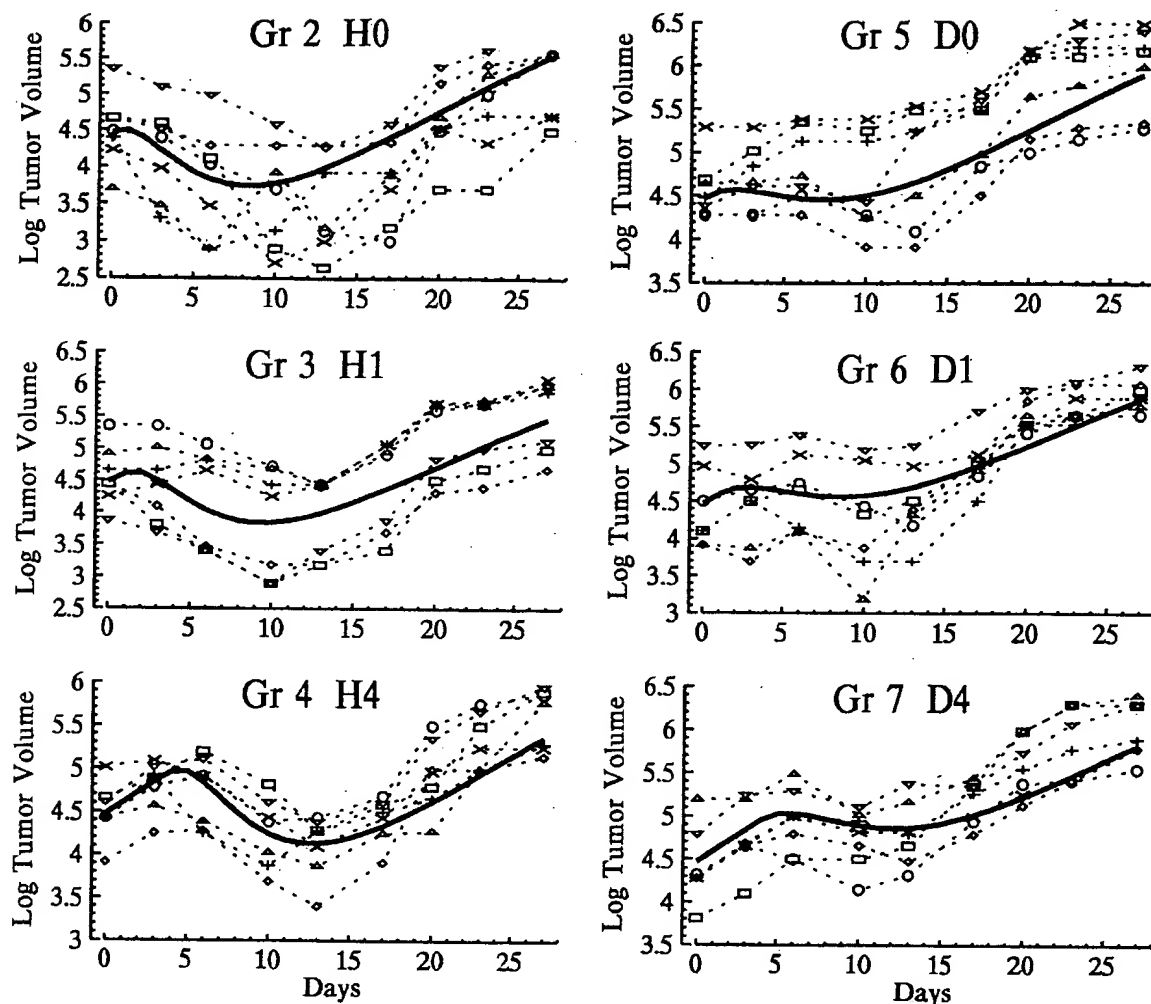Abbreviations: T, trastuzumab; D, doxorubicin.

Fig 2. Tumor volumes for groups receiving single-agent doxorubicin or trastuzumab at the times indicated in Table 3. Symbols connected by dashed lines are the observed natural log of tumor volumes for each mouse. The solid line is the expected trajectory for the group predicted by the growth/drug-effect model. (Reprinted with permission.[7])

This experiment was designed to address the issue of scheduling for trastuzumab/chemotherapy drug combinations. The treatment schedules for this preclinical experiment are shown in Table 3. A differential equation model was developed to describe the growth characteristics of each single agent according to its timing of administration. The observed versus expected trajectories of each individual mouse xenograft are shown in Figure 2. Expected trajectories for drug combinations and schedules predicted by the model were then calculated under the null hypothesis of additivity, and significant departures from the expected tra-

jectories were characterized as either synergistic (if observed antitumor effects were greater than expected) or antagonistic (if results were inferior to the most active single agent alone). The results of this analysis predict that the scheduling of trastuzumab and chemotherapy is important for maximal efficacy, such that schedules utilizing trastuzumab followed by chemotherapy, or concomitant administration of trastuzumab and chemotherapy, are superior to schedules involving chemotherapy followed by trastuzumab (data not shown).[23] Similar scheduling effects have been observed with trastuzumab/cisplatin combinations.[2]

SUMMARY AND CONCLUSIONS

We have shown that preclinical models may be useful for the clinical development of novel antitumor drug combinations, particularly those involving trastuzumab. These models proved important throughout the clinical development of trastuzumab by allowing clinical investigators to choose appropriate dosage and dosing schedules of trastuzumab combinations with chemotherapeutics, which would maximize clinical efficacy. Our model-based approach to drug development was validated by the results from the recent randomized controlled trial of chemotherapy alone versus chemotherapy plus trastuzumab in which patients treated with trastuzumab/chemotherapy combinations had a significant survival advantage compared to patients treated with chemotherapy alone.[10] It is noteworthy that in this trial, the most active synergistic trastuzumab/chemotherapy combinations were not used.[9] Too often, new oncologic drug combinations are developed based on historical empirical chemotherapy recipes, or driven by marketing forces, rather than carefully controlled scientific studies. We propose a rational alternative through the use of appropriate preclinical models. We have seen early evidence of success in the clinic based on our preclinical data. For example, recent studies indicate that combinations of trastuzumab and vinorelbine have extraordinary response rates in metastatic breast cancer,[11] and early results from ongoing trastuzumab/docetaxel and trastuzumab/docetaxel/platinum combinations appear particularly promising (H. Burris, personal communication, December 1999).[12] In particular, trastuzumab/docetaxel/platinum combinations, which appear highly synergistic in our laboratory models, may offer a nonanthracycline alternative to therapy for HER-2–overexpressing breast cancers, which would completely avoid the issues of trastuzumab/anthracycline-associated cardiotoxicity.[12] Clinical trials are currently underway to test this hypothesis.

Treatment of human cancers requires new approaches designed to maximize antitumor efficacy while minimizing toxicity. Therapy directed at specific molecular alterations unique to tumor cells, such as trastuzumab, could prove a more rational approach compared to more conventional, nonspecific cytotoxic anticancer drugs.[2] Integration of such targeted agents with existing therapies in breast cancer is a formidable clinical challenge. However, with the advent of more useful preclinical models, such as those we have developed for trastuzumab combinations, the challenge may be made less difficult with the added potential to find combinations with superior clinical efficacy.[1,2,5,7,8]

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Rationale for Trastuzumab (Herceptin) in Adjuvant Breast Cancer Trials

Dennis Slamon and Mark Pegram

The discovery of the HER2/neu proto-oncogene and its role in the pathogenesis of breast cancer tumors, and the development of the anti-HER2 monoclonal antibody, trastuzumab (Herceptin; Genentech, South San Francisco, CA), directed against the HER2 receptor represent major milestones in the research developments in breast cancer, making trastuzumab the first monoclonal antibody available for treatment of this disease. Clinical trials in HER2-positive patients have demonstrated that the combined use of targeted therapy with trastuzumab in conjunction with cytotoxic chemotherapy is associated with improved time to disease progression and overall survival. Unfortunately, findings also demonstrate an increased risk for cardiotoxicity when trastuzumab is combined with anthracyclines. For HER2/neu-overexpressing breast cancer patients, the adjuvant use of trastuzumab will become paramount; therefore, it must be evaluated in a randomized controlled trial. There is disagreement regarding the design of such a trial, largely because of the ubiquitous use of anthracyclines in the adjuvant setting and the opposing necessity of avoiding anthracycline plus trastuzumab combinations. Combination index values for various chemotherapeutic drugs in combination with trastuzumab demonstrate dramatic synergistic interactions with the platinum agents and with docetaxel (Taxotere; Aventis Pharmaceuticals, Inc, Parsippany, NJ). The greatest level of synergy has been demonstrated with the triple-drug combination of docetaxel, platinum, and trastuzumab in which synergy is demonstrated, even at low doses. The adjuvant trial design for the Breast Cancer International Research Group uses a control arm of doxorubicin/cyclophosphamide for four cycles followed by docetaxel for four cycles and the second arm contains the addition of trastuzumab to the taxane sequence. The third arm, a non–anthracycline-containing regimen, contains docetaxel, a platinum agent (either cisplatin or carboplatin), and trastuzumab. The rationale for the selection of this three-drug regimen is based on the biology of the system and preclinical and clinical findings that demonstrate a high potential for clinical synergy.
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T HE DISCOVERY of the HER2/neu proto-oncogene and its role in the pathogenesis of breast cancers, and the development of the anti-HER2 monoclonal antibody, trastuzumab (Herceptin; Genentech, South San Francisco, CA), directed against the HER2 receptor serve as a useful paradigm for oncology research strategies for the future. In contrast to previous initiatives that combined multiple chemotherapeutic agents in an effort to inhibit solid tumor growth, the use of trastuzumab is a targeted therapy, based on the understanding of growth regulatory processes at the molecular level of the cell. The development of more specific and less toxic therapeutic agents such as trastuzumab will likely be the direction taken for research initiatives in the future.

BACKGROUND

The HER2/neu protein is a member of the type-1 receptor tyrosine kinase family.[1] It is a 185-kd surface membrane protein that is encoded by the HER2/neu gene (also known as the c-erbB-2 gene), which has been localized to chromosome 17q21. Under normal circumstances, the receptor is expressed in a wide variety of tissues, including breast, ovary, endometrium, lung, liver, gastrointestinal tract, kidney, and central nervous system.[2-4] In breast cancer, the HER2/neu gene is amplified in 20% to 25% of all cases, so that instead of having two copies of the gene per cell (one on each chromosome 7) there may be as many as 50 to 100 gene copies per cell.[2,5,6] This amplification of the gene results in overexpression of the gene product, the HER2/neu receptor. In normal tissues, as well as in breast cancer cases that do not have this alteration, the usual number of receptors per cell ranges from 20,000 to 50,000. For tumors that have the alteration, as many as two million receptors per cell, a two-log increase, may be observed.

Tumors with HER2 receptor overexpression are associated with different clinical behavior compared with non-overexpressing tumors. Patients with tumors that display HER2/neu overexpression have a shortened disease-free and overall survival.[2,5]

HER2/neu-negative breast cancer patients demonstrate a median survival of 6 to 7 years compared with HER2-positive patients who have a median survival of 3 years. These findings have been confirmed in larger studies published in the last several years.[7-9] Early on, these clinical data were controversial, in large part due to different methodologies and techniques for detecting HER2 overexpression. To date, the most reliable technology for identifying specific gene amplification is known as fluorescence in situ hybridization.

PROGNOSTIC SIGNIFICANCE

Initially, two explanations for the prognostic significance of HER2 overexpression were hypothesized. The first was that overexpression is an epiphenomena that would allow it to be a useful prognostic marker but not useful as a therapeutic target. The second hypothesis was that the association between HER2 amplification/overexpression and clinical outcome was the result of a direct causal role in pathogenesis. In such a cause, the HER2 receptor could serve as a therapeutic target.

To test the potential role of HER2 overexpression in altering the biological activity of normal and malignant human breast epithelial cells, a number of in vitro studies were conducted in which single-copy, low-expressing cell lines were converted to multiple-copy, high-expressing cells. The biological effects of HER2 overexpression were then measured, including effects on DNA synthesis, cell growth, anchorage-independent growth, tumorigenicity, and metastatic potential. Overexpression of HER2 resulted in an increase in those parameters in malignant cell lines as well as nontransformed immortalized breast cell lines. The data indicate that HER2 overexpression plays a direct role in pathogenesis of breast cancers and therefore would be a useful target.

THERAPEUTIC MODELS

The reagents to study gene products at the time the initial observations on HER2 overexpression were made were monoclonal antibodies directed against the protein receptor. Antibodies were not initially developed as therapeutic agents; however, early results on receptors other than HER2/neu suggested that antibodies could have an impact on tumor growth when directed against the extracellular domain of surface cell receptors.

Several antibody reagents directed against the HER2 extracellular domain were tested. Few antibodies were found to suppress the biological effects induced by HER2 overexpression both in vitro and in vivo in animal models of human breast cancer cells. Complete inhibition of HER2-overexpressing tumor growth was demonstrated after cell exposure to one of these HER2/neu antibodies. Removal of the antibody led to tumor regrowth, indicating that the effects were cytostatic, not cytocidal. The suppressive effects were specific to cells and tissues overexpressing the HER2 gene and were absent from non–HER2-overexpressing cells.

COMBINING HER2/NEU ANTIBODIES WITH CHEMOTHERAPY

One popular method of rendering antibodies cytotoxic envisioned by researchers in the anticancer antibody field was to covalently link cytotoxic drugs to therapeutic monoclonal antibodies. In one such experiment antibodies against the epidermal growth factor receptor (HER1) were coupled to cisplatin and tested against epidermal growth factor receptor-overexpressing head and neck cancer xenografts in vivo.[10]

As controls, the investigators of this study also tested uncoupled free antiepidermal growth factor receptor antibodies given concomitantly with free cisplatin. The control in this experiment demonstrated that concurrent coadministration of the chemotherapeutic agent with the monoclonal antibody resulted in synergistic levels of tumor inhibition, in contrast to physically coupling the agents together, which resulted in only additive levels of tumor inhibition.

Application of findings from the head and neck carcinoma cell line led to investigation of the concurrent coadministration of the HER2 antibodies with cisplatin. Concurrent administration resulted in a significant 100-fold increase in cell kill in HER2/neu-positive cells compared with cisplatin administration alone. No toxicity to other cells was seen, demonstrating specific activity against HER2-positive cells.

We used the method of Chou and Talalay[11] to investigate the potential interaction between other chemotherapeutic agents and the HER2 antibody. This method was chosen because it is the most-referenced and most thoroughly reviewed scientific method used to evaluate drug interactions. The data analysis provides a combination

Table I. Combination Index Values for Chemotherapeutic Drugs and Recombinant Human Monoclonal Antibody HER-2 Combinations In Vitro

Drug	Combination Index	P Value	Interaction
Cisplatin	0.56 ± 0.15	.001	Synergy
Etoposide	0.54 ± 0.15	.0003	Synergy
Thiotepa	0.67 ± 0.12	.0006	Synergy
Docetaxel	0.70 ± 0.16	.003	Synergy
Doxorubicin	1.16 ± 0.18	.13	Addition
Paclitaxel	0.91 ± 0.23	.21	Addition
Methotrexate	1.35 ± 0.17	.21	Addition
Vinblastine	1.09 ± 0.19	.26	Addition
5-Fluorouracil	2.87 ± 0.51	.0001	Antagonism

index value, which describes how the drugs interact. A combination index of 1 is indicative of an additive interaction, whereas a combination index of less than 1 means that the interaction is synergistic. If the combination index is more than 1, the interaction is antagonistic.

Table 1 presents the combination index values for the combinations of various chemotherapeutic drugs and trastuzumab.[12] One of the most dramatic interactions seen with trastuzumab is with the platinum agents, cisplatin or carboplatin, in which a significant degree of synergy is noted. Etoposide, thiotepa, docetaxel, and vinorelbine also result in activity in the synergistic (<1) range. Additive interactions were demonstrated with doxorubicin, methotrexate, and vinblastine. Antagonist interactions (less than additive) are seen with 5-fluorouracil.

Both of the taxanes, paclitaxel (Taxol; Bristol-Myers Squibb Oncology, Princeton, NJ) and docetaxel (Taxotere; Aventis Pharmaceuticals, Inc, Parsippany, NJ), have been assessed in combination with trastuzumab. Paclitaxel resulted in additive activity in combination with trastuzumab, a finding that was corroborated in a study reported by Baselga et al.[13] In contrast, docetaxel resulted in synergistic activity. Repeated study assessments of the combination of docetaxel and trastuzumab consistently demonstrate synergy in the nearly 20 separate experiments performed. While the taxanes have the same basic mechanism of action, paclitaxel and docetaxel demonstrate very different behaviors at the molecular level. Ongoing research is being conducted to determine what these differences may be.

MECHANISM FOR TRASTUZUMAB PLUS PLATINUM/DOCETAXEL SYNERGY

The mechanism for the high level of synergy noted with the platinum salts and trastuzumab has been recently investigated. Platinum induces cell damage by forming adducts in DNA. However, alterations in growth factor receptor pathways may result in a transient decrease in DNA repair that can increase the efficacy of platinum salts. Research conducted at the University of California Los Angeles, and confirmed elsewhere, evaluated cells with and without trastuzumab that had been treated with platinum. Cells without trastuzumab exposure repair much of the platinum damage. However, exposure to trastuzumab attenuates the repair mechanism. Virtually the same data are demonstrated with carboplatin. These data have been confirmed in independent laboratories and in independent studies.

One of the most remarkable findings is the combination index value of the docetaxel, carboplatin/cisplatin, and trastuzumab regimen. Even at the lowest doses, combination indices of less than 0.1 are demonstrated (Table 2).

CLINICAL TRIAL DATA

The initial anti-HER2 antibody to enter clinical trials was a murine monoclonal antibody. A phase I clinical trial conducted at the University of California Los Angeles evaluated the murine monoclonal antibody in patients who were HER2 overexpressors and who had failed multiple chemotherapeutic regimens.[14] Very low doses of the murine monoclonal antibody were initially used, followed by dose escalation. A maximum tolerated dose was never achieved because the only adverse event seen was fever, which developed in 15% of

Table 2. Multiple Drug Effect Analysis of Docetaxel/Carboplatin/Trastuzumab Combinations

Fractional Growth Inhibition	Combination Index	Interaction
0.34	0.083	Synergy
0.38	0.149	Synergy
0.45	0.246	Synergy
0.61	0.322	Synergy
0.63	0.610	Synergy
0.82	0.641	Synergy

patients. Efficacy evaluation revealed that the mouse monoclonal antibody was localized to the tumor and a response was demonstrated in two of 20 heavily pretreated patients. Despite the promising antitumor activity, long-term administration was limited by immune responses to the non-human protein. Patients developed human anti-mouse antibodies against the mouse protein, resulting in rapid clearance of the antibody from the bloodstream and inactivation on subsequent infusions. This necessitated humanization of the antibody, converting the mouse antibody into a human antibody by a combination of chimerization and site-directed mutagenesis. The resultant antibody is 95% human and 5% murine, with the murine portion specifically directed against the human receptor. The humanized anti-HER2 monoclonal antibody is the drug known as trastuzumab and is produced by a genetically engineered Chinese hamster ovary cell line. Recombinant humanized monoclonal antibody HER2 maintains specificity to HER2.

Phase I and Phase II Trials of Trastuzumab

Three phase I studies have been performed to establish the safety and efficacy of trastuzumab.[14] Two of the trials evaluated single-agent trastuzumab; the third trial evaluated trastuzumab in combination with cisplatin. In the first trial, 16 patients received a single fixed dose of trastuzumab ranging from 10 to 500 mg. In the second trial, 17 patients received repeated fixed doses of trastuzumab, also ranging from 10 to 500 mg. Trastuzumab treatment was well tolerated, with no patients experiencing grade 3 or 4 drug-related toxicity. Most of the adverse events reported were mild to moderate in severity, with fever and chills most commonly reported. No patients developed antibodies against trastuzumab. Pegram et al[15] conducted a phase I dose-escalation trial to determine the safety and tolerability of increasing doses of trastuzumab in combination with cisplatin: trastuzumab at a dose of 10, 50, 100, 250, or 500 mg/wk intravenously × 9 weeks and cisplatin 100 mg/m^2 on days 1, 29, and 57. Although the small number of patients and the lack of randomization preclude definitive conclusions regarding tumor response, four of six patients who received either 250 or 500 mg of trastuzumab in combination with cisplatin demonstrated a partial response. Concurrent cisplatin administration did not affect the pharmacokinetics of trastuzumab. No clear relationship between trastuzumab and the incidence of adverse events, which were generally those attributed to cisplatin administration, were noted. Finally, no patients developed antibodies against trastuzumab.

Although not designed to evaluate response, the phase I efficacy findings were notable in that a number of the patients had not previously responded to other therapies. These initial studies provided the rationale for studying trastuzumab as a single agent and in combination with chemotherapy in phase II and III clinical trials in patients with HER2-overexpressing metastatic breast cancer (MBC).

A phase II trial was conducted by Baselga et al[16] in 46 patients with advanced MBC with HER2-overexpressing tumors. Patients were treated with a trastuzumab loading dose of 250 mg on day 0, followed by a weekly dose of 100 mg beginning on day 7 and continuing for a total of 10 doses. At the end of this treatment phase, patients with minor, partial, or complete responses and patients with stable disease were entered onto a maintenance phase of weekly trastuzumab until disease progression. After the first phase of treatment, 43 patients were assessable for response. The overall response rate (complete plus partial responses) was 11.6%, with an additional 37% of patients with minimal response or stable disease. Although the overall response was modest, the data helped confirm that the drug has activity in refractory patients.

A phase II trial conducted by Pegram et al[15] evaluated trastuzumab and cisplatin in combination in 39 MBC patients. To be eligible for participation in this trial, patients had to be HER2 positive, have failed two or more regimens in the metastatic setting, and have objectively demonstrated growth of their tumor of ≥25% while on an active regimen (a chemotherapy-resistant tumor). Patients were treated with an intravenous loading dose of trastuzumab 250 mg on day 0, followed on day 7 by 100 mg/wk × 8 weeks. Cisplatin 75 mg/m^2 was administered intravenously on days 1, 29, and 57. Patients responding to therapy as assessed on day 70 were entered onto a maintenance program consisting of trastuzumab 100 mg/wk plus cisplatin 75 mg/m^2 every 4 weeks until progressive disease or unacceptable toxicity. Despite the strict entry criteria, the results reveal a complete and partial response rate of 24% in 37

assessable patients. An additional 24% of patients had a minor response or stable disease. The regimen was well tolerated overall, with no indication that trastuzumab potentiates cisplatin toxicity. The safety and efficacy profile demonstrated in these trials led to the pivotal phase III trial of trastuzumab in large numbers of patients.

Pivotal Phase III Clinical Trial

The phase III pivotal clinical trial was designed to compare trastuzumab/chemotherapy combination with chemotherapy alone.[17] The choice of chemotherapy was considered best available standard therapy for first-line MBC. At the time of trial design in 1995, this was defined as doxorubicin (or epirubicin in participating Canadian and European countries) plus cyclophosphamide (AC). The primary end points were time to disease progression and safety. Secondary end points were overall response rate, response duration, time to treatment failure, and survival. The eligibility criteria included measurable MBC, HER2 positivity, no prior chemotherapy, and a Karnofsky performance status of better than 60%. Patients were randomized to receive doxorubicin 60 mg/m^2 (or epirubicin 75 mg/m^2) plus cyclophosphamide 600 mg/m^2 every 3 weeks with or without trastuzumab for six cycles. Women with prior anthracycline exposure were randomized to a paclitaxel arm: 175 mg/m^2 every 3 weeks with or without trastuzumab. Four hundred fifty patients were needed for randomization; 469 patients were enrolled. The patient characteristics were well balanced for chemotherapy alone versus chemotherapy plus trastuzumab. Sixty percent (60%) of patients were in the anthracycline subgroup and 40% were in the paclitaxel subgroup.

Results demonstrated improvement of overall response rate by 53% and improvement in response duration by 58% when trastuzumab was used in combination with chemotherapy versus chemotherapy alone. In addition, the primary end point, time to disease progression, was improved by 65% in patients receiving trastuzumab in combination with chemotherapy versus chemotherapy alone. Additional analysis demonstrates that the response differences were even more apparent in the paclitaxel/trastuzumab subgroup of patients compared with the paclitaxel-alone subgroup. This was accounted for by the low response rate in the paclitaxel-alone arm.

Moreover, a survival advantage for patients treated with trastuzumab/chemotherapy compared with chemotherapy alone was demonstrated. The 1-year results demonstrated a 67% survival rate for the patients treated with chemotherapy alone versus 78% for those treated with trastuzumab/chemotherapy. These findings were statistically significant ($P < .05$). The updated results over a 29-month follow-up period demonstrate that the difference in survival is associated with an approximately 25% decrease in the relative risk of death.

Toxicity Profile of Trastuzumab Plus Chemotherapy

Before the completion of the pivotal trial, trastuzumab appeared to be phenomenally nontoxic in all parameters, even when combined with chemotherapy. There were no significant increases in any kind of toxicity except for fevers and rigors that occurred with initial infusion and that could be easily controlled. However, completion of the phase III trial demonstrated a dramatic increase in cardiotoxicity when trastuzumab is used simultaneously with chemotherapy, particularly when used in combination with an anthracycline (Table 3). Cardiac dysfunction, defined as New York Heart Association class I through IV, occurred in 27% of patients receiving trastuzumab plus AC versus 6% receiving AC alone, accounting for a fourfold increase. The incidence of New York Heart Association class III and IV cardiac dysfunction was higher among patients receiving trastuzumab plus AC than in those receiving AC alone (16% v 3%).

A study conducted by Vogel et al[18] allows ex-

Table 3. Cardiac Dysfunction Outcomes Using Trastuzumab in Combination With Chemotherapy

	H + AC	AC	H + T	T
No. of cardiac dysfunction events	39	9	11	2
No. of events after trastuzumab treatment	14	5*	6	1*
No. of deaths	4	1	1	2
MBC	4	0	0	2
Cardiac	0	1	0	0
Pneumonia	0	0	1	0

Abbreviations: H, trastuzumab; AC, doxorubicin/cyclophosphamide; T, paclitaxel.
* Trastuzumab extension protocol.

amination of the possibility that trastuzumab alone is the cause of cardiotoxicity. One hundred thirteen women with MBC were treated with single-agent trastuzumab. Cardiotoxicity was documented in only two patients (1.7%), therefore discounting the possibility that the drug per se is cardiotoxic. It is interesting that the preclinical mouse model did not predict for this interaction because trastuzumab does not work against the mouse heart muscle receptor; rather, trastuzumab is specific for the human receptor. Ongoing research is investigating the mechanism of trastuzumab/anthracycline cardiotoxicity.

TRASTUZUMAB IN THE ADJUVANT SETTING

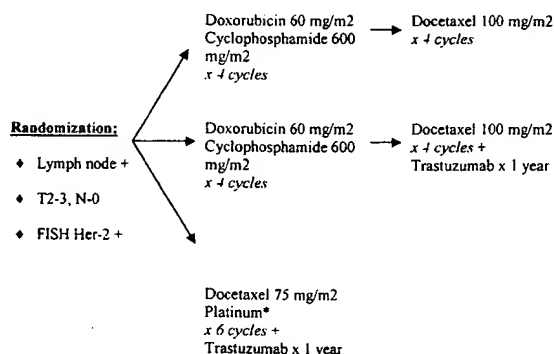For HER2/neu-overexpressing breast cancer patients it is paramount that the adjuvant use of trastuzumab be evaluated in a randomized controlled trial. Disagreement regarding the design of such a trial exists, largely because of the ubiquitous use of anthracyclines in the adjuvant setting and the opposing necessity of avoiding anthracycline/trastuzumab combinations in the adjuvant and metastatic settings. The trial design for the Breast Cancer International Research Group (BCIRG) study group uses a control arm that is the current standard of care in the United States: AC for four cycles followed by a taxane for four cycles. In this case, the taxane will be docetaxel (Fig 1). The second arm would be identical, with the addition of trastuzumab to the taxane sequence. A third arm would be a non–anthracycline-containing regimen; the proposed regimen based on biologic

rationale is docetaxel, a platinum agent (either cisplatin or carboplatin), and trastuzumab (TCH). The rationale for the selection of this three-drug regimen is not empiric, but rather is based on the biology of the system and preclinical and clinical findings.

Although data exist regarding the safety and efficacy of the trastuzumab/platinum, trastuzumab/docetaxel, and platinum/docetaxel combinations, data are not yet available regarding the safety and efficacy of the three-drug TCH regimen. To ensure the safety of the TCH regimen, two parallel phase II pilot studies are ongoing: a study of the docetaxel/carboplatin/trastuzumab combination in the United States and a study of the docetaxel/cisplatin/trastuzumab combination in Canada and Europe. As entry criteria in these phase II studies and for the pivotal adjuvant study, the fluorescence in situ hybridization technique will be used because it is the most accurate technique currently available for the detection of HER2/neu gene alteration.

CONCLUSION

Advantages to the proposed BCIRG study are the direct comparison of the TCH regimen to the standard anthracycline-containing regimen of AC followed by docetaxel. If cardiotoxicity should occur in the anthracycline-containing study regimen, this arm can be closed without discontinuing the entire study. Therefore, the BCIRG study will provide results on the adjuvant use of trastuzumab in the occurrence of anthracycline-induced cardiotoxicity. An additional advantage of the BCIRG adjuvant study is the ability to evaluate a non–anthracycline-regimen that is based on a strong biologic rationale that renders the potential for a very significant therapeutic improvement in breast cancer treatment. Given the potential for cardiotoxicity, investigation of only anthracycline-based regimens in combination with trastuzumab may be risky and time consuming. One must be willing to overcome the paradigm that anthracyclines must be applied in the adjuvant setting. For HER2-overexpressing breast cancer patients, it could be particularly true that the biology of their tumor makes them well suited for treatment with a taxane/platinum/trastuzumab-based regimen. Adjuvant trials involving such a combination of agents hold future promise for patients with HER2-overexpressing breast cancer.

Randomization:

- ◆ Lymph node +
- ◆ T2-3, N-0
- ◆ FISH Her-2 +

Doxorubicin 60 mg/m2
Cyclophosphamide 600 mg/m2
x 4 cycles → Docetaxel 100 mg/m2 x 4 cycles

Doxorubicin 60 mg/m2
Cyclophosphamide 600 mg/m2
x 4 cycles → Docetaxel 100 mg/m2 x 4 cycles + Trastuzumab x 1 year

Docetaxel 75 mg/m2
Platinum*
x 6 cycles + Trastuzumab x 1 year

*Cisplatin 75 mg/m2 or Carboplatin AUC = 6

Fig 1. Randomized adjuvant phase III study of HER2-positive patients (BCIRG study 006).

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Preclinical Studies of Gemcitabine and Trastuzumab in Breast and Lung Cancer Cell Lines

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Abstract

Overexpression of the HER2/*neu* oncogene and receptor protein has been reported in 20%-30% of patients with breast cancer and is associated with a poor prognosis. HER2/*neu* expression in breast cancer patients assessed by fluorescence in situ hybridization or immunohistochemistry is a predictor for response to trastuzumab, a humanized monoclonal antibody against the HER2/*neu* cell-surface protein. Data regarding HER2/*neu* expression in lung cancer are more limited, and there is little information regarding HER2/*neu* expression and response to trastuzumab alone or in combination with chemotherapeutic agents. Gemcitabine is an active agent against non–small-cell lung cancer (NSCLC) and has demonstrated activity in breast cancer as well. In vitro modified tetrazolium salt growth assays were performed to determine whether the combination of trastuzumab/gemcitabine produced synergistic or additive effects on breast and lung cancer cell lines. The effects of trastuzumab alone, gemcitabine alone, and the trastuzumab/gemcitabine combination was evaluated on 4 NSCLC cell lines, 1 small-cell lung cancer (SCLC) cell line, and 2 breast cancer cell lines. HER2/*neu* surface protein expression was assessed by fluorescence flow cytometry and immunohistochemistry. Fluorescence in situ hybridization analysis was used to study gene expression. Trastuzumab treatment alone resulted in growth inhibition in all cell lines expressing HER2/*neu* and the inhibitive effect correlated with the level of cell surface HER2/*neu* protein expression. Treatment with gemcitabine alone resulted in growth inhibition in both breast and NSCLC cell lines. A synergistic growth inhibition effect was seen with the trastuzumab/gemcitabine combination as indicated by combination index values < 1. The degree of synergy observed did not directly correlate with the level of surface protein expression, as synergy was seen even in cancer cell lines expressing low levels of HER2/*neu*. No treatment effect was seen in the SCLC cell line, which did not express HER2/*neu*. These preclinical studies indicate a need to study the clinical synergistic effects of the gemcitabine/trastuzumab combination in breast cancer and NSCLC patients whose tumors overexpress HER2/*neu*.

Introduction

Lung cancer is the leading cause of cancer death in the United States and the Western world. Lung cancer kills more individuals than breast, colorectal, and prostate cancers combined.[1] The 5-year survival rate remains < 15%, despite advances in combined treatment modalities.[2] New tar-

geted therapies have been developed based on advances in molecular biology. They appear to be more tumor specific and less toxic than standard chemotherapeutic agents. Many of these agents are already in clinical trials, and some have been approved by the US Food and Drug Administration. These developments provide potential for better treatment of lung cancer with the prospect of reduced toxicity and significantly improved survival.

In lung cancer, overexpression of dominant oncogenes due to gene amplification, increase in chromosome copy numbers, transcriptions, and other means has been reported to occur frequently.[3] The *erbB* (HER) family of oncogenes is frequently overexpressed in lung cancer tumors.[4] The *erbB* gene family codes for growth tyrosine kinase receptors that

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appear to play an important role in the autocrine growth of human lung cancer.[5-9] The family consists of 4 receptors: erbB-1 (HER1), erbB-2 (HER2/neu), erbB-3 (HER3), and erbB-4 (HER4).

Recognition of the role of the HER2/neu signaling pathway in breast cancer led to the development of a new treatment strategy based on interference of this pathway. In preclinical studies, trastuzumab, a humanized monoclonal antibody raised against the HER2/neu surface receptor, inhibited growth of human breast cancer cells when used alone and in combination with some chemotherapeutic agents.[10] In subsequent clinical trials, trastuzumab produced objective responses in about 20% of the patients with HER2/neu-overexpressing tumors (defined by immunohistochemistry

Table 1 HER2 Protein Overexpression and Gene Amplification in Cell Lines[22]						
	HER2 Overexpression			HER2 Gene Amplificaiton		
Cell Line	HER2+ (%)	MFI	IHC	Average HER2 Genes per Cell	Maximum HER2 Genes per Nucleus	Ratio of Chromosomes to Gene
NSCLC						
Calu-3 Adenocarcinoma	100	36.0	3	30	64	> 2
NCI-H322 Adenocarcinoma	98	9.8	2	6.5	14	1.1
A549 Adenocarcinoma	72	3.7	0	3.7	8	1.0
NCI-H460 Large cell	57	2.4	0	3	6	1.0
SCLC						
SHP77	0	0	0	2	4	0.7
Breast Cancer						
SK-BR-3	100	43.0	3	50	80	> 2
MCF-7	98	10.8	3	2	5	0.7

Abbreviations: IHC = immunohistochemistry; MFI = mean fluorescence intensity; NSCLC = non–small-cell lung cancer; SCLC = small-cell lung cancer

[IHC]).[11] Clinical synergy was clearly demonstrated when trastuzumab and conventional chemotherapeutic agents were used in combination in breast cancer patients, confirming results of preclinical studies. The combination of trastuzumab with doxorubicin- or paclitaxel-based chemotherapy produced higher response rates and longer survival than either agent alone.[12]

Studies of HER2/neu expression and gene amplification in lung cancer have lagged behind those in breast cancer. Findings from previous studies suggest that overexpression of HER2/neu in non–small-cell lung cancer (NSCLC) implies poor prognosis (as in breast cancer); overexpression occurs in the tumors of approximately 20% of NSCLC patients, as evaluated by IHC techniques.[13-21] However, more recent studies of NSCLC tumors have shown that in contrast to breast cancer, very few malignant lung tumors have true gene amplification[21,22]; and increased protein expression in lung cancer is usually moderate (1+/2+ by DAKO HercepTest™; DAKO Corporation, Carpinteria, CA) and most frequently is due to balanced chromosome/gene copy duplications.[21]

Based on the clinical experience in breast cancer, it was logical to determine whether the combination of trastuzumab with traditional chemotherapeutic agents would lead to synergistic growth inhibition in lung cancer as well. Gemcitabine is among the new chemotherapeutic agents with a high level of activity in NSCLC[2] and demonstrated activity in breast cancer. It was therefore logical to conduct preclinical studies of trastuzumab and gemcitabine and combinations with these agents to determine whether a synergistic treatment effect resulted with the combination. This report

summarizes such preclinical studies that have been conducted at the University of Colorado Cancer Center.

Studies on Lung and Breast Cancer Cell Lines

Methods

At the University of Colorado Cancer Center, we studied surface protein HER2/neu expression in 19 NSCLC, 3 small-cell lung cancer (SCLC), and 5 breast cancer cell lines by using fluorescence flow cytometry and IHC to compare results of these methods. For fluorescence-activated cell sorter (FACS) analysis, cells ($5x10^5$) were incubated with the monoclonal antihuman HER2/neu antibody c-erb-2 Ab-2 (9G6.10; NeoMarkers, Inc, Fremont, CA) or the isotype matched control, MOPC 10 (Sigma Chemical Co, St. Louis, MO).[22] The HercepTest™ was used for IHC analysis and results were scored from 0-3+ according to standard protocol. HER2/neu gene expression was analyzed by fluorescence in situ hybridization (FISH) using a dual color technique (PathVysion™ HER2 DNA probe kit; Vysis, Inc, Downer's Grove, IL).

For preclinical therapeutic studies with single-agent trastuzumab, single-agent gemcitabine, and the 2 drugs combined, 4 NSCLC cell lines (Calu-3, NCI-H322, A549, and NCI-H460), 1 SCLC cell line (SHP77), and 2 breast cancer cell lines (SK-BR-3, MCF-7) were studied for cell-surface expression of HER2/neu. The cell lines and culture conditions are described in detail elsewhere.[22] To assess cell growth inhibition by trastuzumab, gemcitabine, and the combination, the modified tetrazolium salt assay was used.[23] When cell

Figure 1 Growth Inhibition by Trastuzumab Alone in NSCLC, SCLC, and Breast Cancer Cell Lines
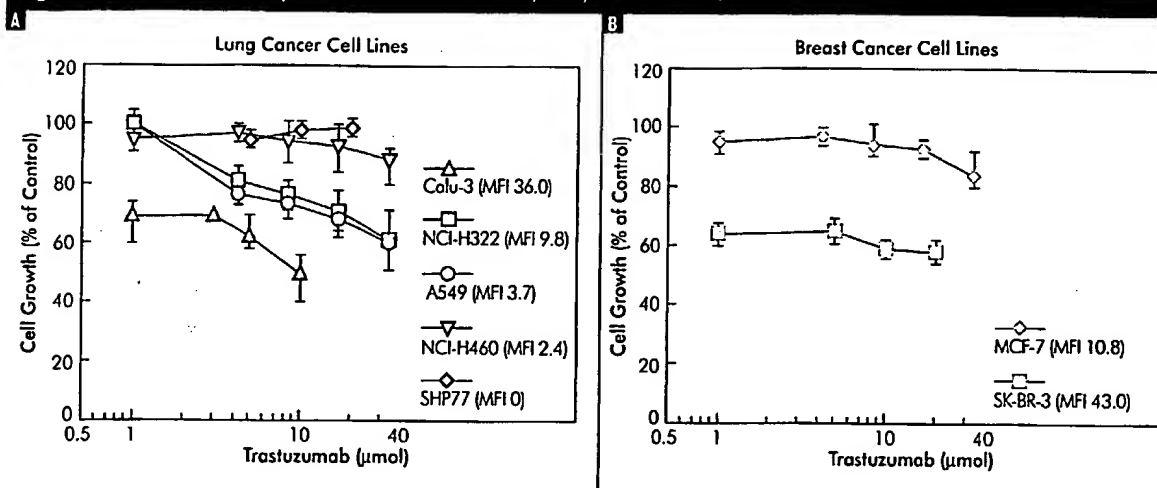
Abbreviations: MFI = mean fluorescence intensity; NSCLC = non–small-cell lung cancer; SCLC = small-cell lung cancer

lines were incubated with combinations of trastuzumab and chemotherapeutic agents, the combination effects were assessed with the isobologram method of Chou and Talalay.[24]

Results

Expression of HER2/neu by Flow Cytometry, Immunohistochemistry, and Fluorescence In Situ Hybridization. A comparison of IHC and FACS showed an excellent correlation (r = 0.57; P =0.002) between the 2 methods in determining cell-surface protein HER2/neu expression. By using criteria of 0 or 1+ HercepTest™ and mean fluorescence intensity (MFI) < 4 as negative for HER2/neu expression, and 2+ or 3+ HercepTest™ and MFI > 6 as positive, 15 cell lines had no expression by either test and 7 cell lines were positive by both methods, while discordant results between the methods were seen in 5 of 27 cell lines.[22]

The mechanism of HER2/neu oncogene overexpression was assessed by FISH in 4 NSCLC cell lines, 1 SCLC cell line, and 2 breast cancer cell lines (Table 1). The NSCLC cell lines (Calu-3) had gene amplification indicated by numerous HER2/neu signals (> 30) within each cluster, which was also found for the breast cancer cell line SK-BR-3. In both cases, FISH results correlated with IHC results of 3+. The NSCLC cell line NCI-H322 had an intermediate MFI of 9.8, corresponding to 2+ on the HercepTest™, and an average of 6.5 genes per cell and maximum 14 genes in one nucleus, while the last 2 NSCLC cell lines and the SCLC cell line had almost no abnormal copies of the gene and no positive IHC staining.

Treatment with Trastuzumab Alone. Growth inhibition induced by trastuzumab alone is shown in Figure 1. The greatest growth inhibition was seen in cell lines expressing high levels of HER2/neu, ie, NSCLC cell line Calu-3 and breast cancer cell line SK-BR-3. Trastuzumab concentrations as low as 0.1 μmol resulted in modest growth inhibition in the 2 cell lines. In contrast, higher trastuzumab concen-

trations were required to inhibit cell lines exhibiting moderate HER2/neu levels, ie, NSCLC cell lines H322 and A549; trastuzumab concentrations greater than 1 μmol were required to produce any growth inhibition, and high concentrations (30 μmol) only partly inhibited growth inhibition of these cell lines. No growth inhibition was seen in the non–HER2/neu-expressing SCLC cell line.

Effects of the Trastuzumab/Gemcitabine Combination on Cell Growth. The isobologram combination index (CI) method of Chou and Talalay[24] was used to quantitate the combination effects of trastuzumab/gemcitabine. A CI < 0.5 indicates highly synergistic effects, while a CI of 0.5-1.0 indicates synergy, and a CI of 1.0-1.2 indicates additive interaction. The data are shown in Figure 2. For the breast cancer cell line SK-BR-3 with high level HER2/neu expression (MFI = 43), synergy to strong synergy was seen for all concentrations of trastuzumab and gemcitabine, while for the breast cancer cell line MCF-7 with low HER2/neu expression (MFI = 10.8), strong synergy was seen only with the high concentrations of gemcitabine and trastuzumab. In the NSCLC cell lines, we observed strong synergy to synergy with all trastuzumab and gemcitabine concentrations; synergy was seen even in A549 (MFI = 3.7) and NCI-H460 cell lines (MFI = 2.4), which do not have high level of HER2/neu expression. However, no synergistic effect was seen in the non– HER2/neu-expressing SCLC cell line.

Conclusion

Preclinical studies to date have shown that trastuzumab treatment alone in HER2/neu-expressing breast and lung cancer cell lines results in significant growth inhibition. However, the growth inhibitory effect correlated with the level of HER2/neu expression, with the most significant effect seen in cell lines with high levels of HER2/neu expression. Gemcitabine treatment alone also resulted in growth

Figure 2 Effects of Trastuzumab/Gemcitabine on Growth Inhibition in Tumor Cell Lines
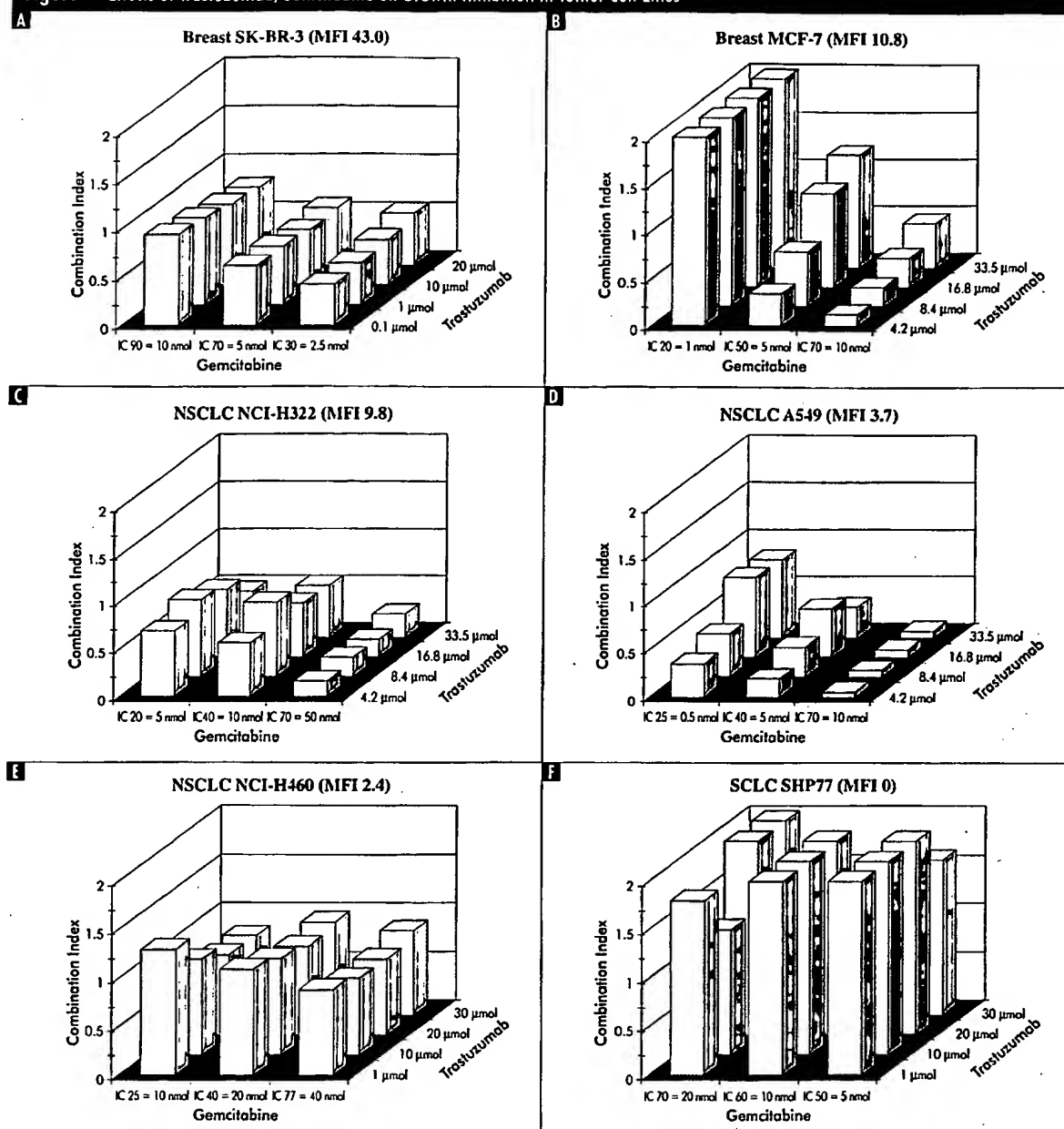
The isobologram combination index (CI) method of Chou and Talalay[24] was used to quantitate the effects of trastuzumab/gemcitabine growth inhibition in tumor cell lines including breast cancer SK-BR-3 (A), breast cancer MCF-7 (B), NSCLC NCI-H322 (C), NSCLC A549 (D), NSCLC NCI-H460 (E), and SCLC SHP77 (F). CI values > 1.2 indicate antagonism, CI values 0.5-1.0 indicate synergy, and CI values < 0.5 indicate strong synergy.

Abbreviations: IC = inhibitory concentration; MFI = mean fluorescence intensity; NSCLC = non-small-cell lung cancer; SCLC = small-cell lung cancer

inhibition in both breast and lung cancer cell lines. Significant synergy was obtained by combining trastuzumab and gemcitabine in both breast and lung cancer cell lines. The synergistic effect with the combination did not correlate with the level of HER2/*neu* expression; strong synergy was even seen in cell lines with low-level HER2/*neu* expression.

However, no synergistic effect was seen in the non–HER2/*neu*-expressing SCLC cell line.

These studies support the study of gemcitabine in combination with trastuzumab in patients with breast cancer and NSCLC. The clinical efficacy of trastuzumab has been clearly demonstrated in breast cancer patients whose tumors overex-

press HER2/neu, and the efficacy of the trastuzumab/gemcitabine combination should be explored in these patients.

Clinical data on NSCLC patients treated with trastuzumab are more sparse. However, gemcitabine is used commonly to treat NSCLC, and preclinical data provide a rationale for evaluating the clinical effect of the trastuzumab/gemcitabine combination. The current preclinical study showed strong synergy between the 2 agents even in NSCLC cell lines with low HER2/neu expression. Thus, clinical synergistic effects of gemcitabine and trastuzumab, and correlation to the level of HER2/neu expression, need to be studied further in NSCLC, using both IHC and FISH analyses.

Randomized clinical phase III trials in breast cancer and NSCLC would be required to clarify the clinical synergistic effect of these agents. Based on the breast cancer experience, studies with NSCLC patients will need to include intergroup participation due to the low frequency of NSCLC patients with HER2/neu gene amplification and IHC 3+ tumors (< 10%).[21] However, synergistic effect between gemcitabine and trastuzumab was also observed in NSCLC cell lines with a low level of HER2/neu expression, indicating that synergy between the drugs might be obtained even in patients with low level HER2/neu-expressing tumors.

Acknowledgement

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